Margaret Pitt[illegible], Ph.D.

COMMISSION ON IMMUNIZATION

Annual Report to the Armed Forces Epidemiological Board

1968 - 1969

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COMMISSION ON IMMUNIZATION

DIRECTOR'S SUMMARY REPORT - FALL 1969

On March 28, 1969, an executive session of the Commission on Immunization was held reviewing the Commission missions and contract applications. Dr. Jonathan W. Uhr was appointed Deputy Director. The annual technical meeting was held on 16-18 October, 1969 at Walter Reed Army Institute of Research as a joint meeting with the Commission on Parasitic Diseases. This was highlighted by a symposium on "Immunity and Parasitic Diseases" on the second day of the meeting. The joint meeting afforded the opportunity for each group to become familiar with each others program and to appreciate the areas of mutual interest. On the third day the Commission on Immunization was afforded the opportunity to become familiar with immunological studies supported by the Medical R & D Research and Development Command which have not been under Commission sponsorship.

I. Annual Meeting

a. Agenda

October 16, 1969

Introductory Remarks - Commission Directors, President and Executive Secretary of the Board

Reports, Preventive Medicine Officers:
Department of the Army
Department of the Navy
Department of the Air Force

Report, Representative of USA Med. R & D Command

Progress Reports on Research Sponsored by Commission on Parasitic Diseases

Dr. Paul C. Beaver: Filariasis in Relation to Tropical Eosinophilia

Dr. Henry van der Schalie: Studies of the Intermediate Snail Hosts of Oriental and African Schistosomiasis

Dr. Franz von Litchtenberg: Protective Mechanisms in Schistosome Infection

Dr. William P. Stucki: Parasite Antigens

Dr. John Janovy, Jr.: Effects of Temperature on *Leishmania* Metabolism

Dr. Donald W. Twohy: The Nature of Immunity to Leishmaniasis

Progress Reports on Research Sponsored by Commission on Immunization

Dr. Geoffrey Edsall: Factors Influencing the Pattern of the Immune Response

Dr. Willard F. Verwey: Effect of Dosage Interval on Response to Reimmunization with Cholera Vaccine

Dr. Karl F. Meyer: Studies on Immunization of Man Against Plague

Dr. Karl F. Meyer: Isolation and Characterization of Lymphogranuloma Venereum Agents

Dr. Abraham G. Osler: Mechanisms of Hypersensitivity

Dr. Jonathan W. Uhr: Antibody Formation and Immunity

Dr. Herman N. Eisen: Antibody Structure and Function

October 17, 1969

Symposium on Immunity and Parasitic Diseases

Structure and Functions of Immunoglobulins - H. N. Eisen

Mechanisms of Allergy and Hypersensitivity - K. F. Austen

Immune Mechanisms of Resistance - G. B. Mackaness

General Discussion

Immunoglobulins and Hypersensitivity in Toxoplasmosis - J.S. Remington

Mechanisms of Histamine Release in Rabbit Schistosomiasis - J. F. Barbaro

Cell Mediated Immune Response - E.J.L. Soulsby

Delayed Hypersensitivity and Lymphocyte Transformation in *Trichinella spiralis* - C. W. Kim

Delayed Hypersensitivity and Granuloma Formation in Schistosomiasis - K. S. Warren

Antigens Common to Host and Parasite - R. T. Damian

Immunization by the Use of Irradiated Parasites - E. H. Sadun

Review of Areas of Interest to Commission on Immunization

Cholera and Typhoid Fever - R. B. Hornick

Smallpox - J. D. Millar

Vaccination - A. S. Benenson

Plague -

Yellow Fever - J. C. Wagner

October 18, 1969

Report of New Work, New Investigators and Guests

Dr. Edgar E. Baker - A Chemical and Biological Study of the Antigens of *Pasteurella pestis*

Dr. Wm. A. Altemeier - The Efficiency of IgM Antibodies to the O Antigen of *S. typhosa*

Dr. Nicholas P. DiLuzio - Role of the Reticuloendothelial System and Opsonins in Malaria and Other Parasitological Infestations

Dr. Bernard B. Levine - Classes and Properties of Human Antibodies

Dr. Noel R. Rose - Cellular and Humoral Immunity in Parasitic Infections.

Dr. Wayne L. Ryan - Macrophage in the Immune Response

Dr. Arthur M. Silverstein - Fetal Response to Ummunization

General Discussion

Executive Session, Commission on Immunization

Adjournment

b. Attendance

Members Commission on Immunization	October 16	17	18
Dr. K. Frank Austen	x	x	x
Dr. Elmer L. Becker			
Dr. Abram S. Benenson	x	x	x
Dr. Geoffrey Edsall	x	x	x
Dr. Herman N. Eisen	x	x	x
Dr. C. Henry Kempe			
Dr. Irwin H. Lepow	x	x	x
Dr. Karl F. Meyer	x	x	x
Dr. Robert Oseasohn		x	x
Dr. Jonathan W. Uhr	x	x	x
Associate Members Commission on Immunization			
Dr. Albert H. Coons		x	
Dr. Thomas J. Gill, III	x	x	x
Dr. William Gochenour, Jr., Col., VC	x		
Dr. Karl Habel			
Dr. Richard B. Hornick		x	
Dr. Samuel L. Katz	x	x	
Dr. George B. Mackaness	x	x	x
Dr. John D. Marshall, Jr.		x	x
Dr. J. Donald Miller	x	x	
Dr. Abraham G. Osler	x	x	x
Dr. Alwin M. Pappenheimer, Jr.			
Dr. Margaret Pittman	x	x	x
Dr. Otto J. Plescia			
Dr. Fred S. Rosen	x	x	x

Associate Members Commission on Immunization	October 16	17	18
Dr. Elvio H. Sadum		x	
Dr. Jay P. Sanford	x	x	
Dr. Willard F. Versey	x		x
Dr. John C. Wagner	x	x	
Responsible Investigators Commission on Immunization			
Dr. William A. Altemeier	x	x	x
Dr. Noel R. Rose	x	x	x

Members Commission on Parasitic Diseases	October 16	17
Dr. Paul C. Beaver	x	x
Dr. Ernest Bueding	x	
Dr. Rodney C. Jung	x	x
Dr. Robert M. Lewert	x	x
Dr. Harry Most	x	
Dr. Lloyd E. Rozeboom	x	x
Dr. Elvio H. Sadun		x
Dr. Leslie A. Stauber	x	x
Dr. Henry van der Schalie	x	x
Dr. Thomas H. Weller	x	x
Dr. Willard H. Wright	x	x
Dr. Martin D. Young	x	x
Associate Members Commission on Parasitic Diseases		
Dr. Robert M. Altman	x	
Dr. Daniel H. Connor	x	x
Dr. John Scanlon	x	x
Responsible Investigators Commission on Parasitic Diseases		
Dr. William L. Hanson	x	x
Dr. John Janovy, Jr.	x	x
Dr. William P Stucki	x	x
Dr. Donald W. Twohy	x	x
Dr. Franz C. von Lichtenberg	x	x
Dr. Kenneth S. Warren		x
Col. Bradley W. Prior, USAF, MC, Exec. Secty.	x	x

Army, Navy, Air Force and Guests	October 16	17	18
Maj. Robert Edelman, Hq, USAMRDC	x	x	x
LTC Dale E. Wykoff, Hq, WRAIR, WRAMC	x		
Col. Lyman P. Frick, Hqs, WRAIR	x	x	
Maj. Craig A. Llewellyn, Prev. Med. Resident, WRAIR	x		
Maj. Seymour H. White, MC, USAMRDC	x	x	
LTC Harry Dangerfield, USAMRIID	x		
LTC Phillip Winter, Prev. Med. Div., OTSG	x	x	x
LTC Donald W. Sample, Hq, USAMRDC	x	x	x
CPT Ray H. Berry, USA WRAIR		x	
Maj. R. Wistar, MC, WRAIR		x	x
CPT William Habig, USAMRIID			x
Cdr. J. S. Cassells, NAU Med Sch, Bethesda	x		
Cdr. Stephen J. Kendra, BUMED	x	x	
LTC Otis W. Jones, Hq, USAF (AFMSPAP)	x	x	
Col. George W. Powell, Hq, USAF (AFMSPAP)	x	x	
Lt. Col. Robert J. Brandt, USAF, MC, Hq, USAF (AFMSPAP)	x	x	
Franklin Neva, Ch, Lab. Para. Dis., NIH, Bethesda	x		
Col. H. W. Whitcher, British Liaison Officer	x	x	
John R. Seed, Assoc. Prof. Tulane University	x	x	
Wayne L. Ryan, Prof. Univ. of Nebraska	x		x
Charles W. Kim, Scientist, Brookhaven Natl Lab, N.Y.	x	x	
Myron Schultz, Ch. Para. Dis. Br., NCDC, Atlanta, Ga.	x	x	
Edgar E. Baker, Prof. Microbiology, Boston Univ. Sch. Med.	x	x	x
Arthur M. Silverstein, Johns Hopkins Med Sch, Baltimore	x	x	x
Bernard B. Levine, Assoc. Prof. N.Y.U. Med. Sch.	x	x	x
S. B. Formal, WRAIR	x	x	
J. F. Barbaro, WRAIR		x	
R. T. Damian, Southwest Foundation		x	
E.J.L. Soulsby, Univ of Pennsylvania		x	
J. S. Remington, Stanford Med. Sch.		x	
A. Stone, PMO, WRAMC		x	
Donald T. O. Wong, WRAIR		x	
P. A. Ward, AFIP		x	
Virginia G. McGlenn, USAMRIID	x		
D.L.Babos, Case Western Reserve Univ., Cleveland, Ohio		x	
Ruth Nussensweig, New York University			
W. P.Stucki, Res. Biochemist, Parke-Davis			x
D. W. Twohy, Assist. Prof., Michigan State University			x
William L. Hanson, University of Georgia			x
James S. Rust, WRAIR			x
N. P. DiLuzio, Prof. Tulane Medical School			x

II. Symposium on Immunity and Parasitic Diseases

The Symposium on Immunity and Parasitic Diseases was held on 17 October. This program had been organized by Dr. Elvio Sadun, working with Drs. Elmer Becker and Peter Ward. An opening portion covering present concepts of immunology was followed by examples of these phenomena in parasitic diseases. Dr. H. N. Eisen opened, describing the chemical structure and function of the immunoglobulin molecule. Dr. K. F. Austen spoke on the mechanisms of allergy and hypersensitivity differentiating between the immediate reactions, which could either be cytotoxic with direct action against some cell surface antigen, or anaphylactic in which the antigen-antibody interaction releases chemical mediators which act at secondary sites. In subacute reactions the immune complex is deposited in the lesion itself which then activates the complement system, attracting polies. Finally, delayed reactions depend on an antigen interacting with sensitized lymphocytes which then produce a mediator chemotactic for mononuclear macrophages. His review of the complement system and the reactions mediated by it served to bring the entire group up to the present in a fast moving field.

Dr. G. B. Mackaness reviewed the immune mechanisms of resistance, reporting on studies in which listeriosis in mice was used as a disease model, as well as BCG. The mechanism as presently conceived starts with an immune response in which lymphoid cells respond to the antigen, giving rise to a population of immunologically committed lymphoid cells. These cells, which on transfer can confer resistance to a second animal, have the capacity to influence the behavior of host mononuclear phagocytes; these are essential for the final resistance. These lymphocytes promote mitoses of the phagocytes; they cause very rapid mobilization of these cells to the site of infection, and they promote the process whereby the mononuclears become activated. The final activity may be due to the release of an intermediate product; this final evoked resistance is not necessarily specific for the original antigen.

Dr. Remington discussed immunoglobulins and hypersensitivity in toxoplasmosis, demonstrating the value of the serological studies for IgM as a method for determining whether an infant has a congenital toxoplasma infection, since on the one hand IgG may be present in the infant's serum as evidence of maternal infection, and on the other hand the majority of congenital infections are not clinically evident. He also presented his studies in which toxoplasma-infected animals proved to be resistant to virus, bacteria and protozoa for as long as one year. Using pyran as an interferon inducer, prolonged resistance against toxoplasma ensued, but interferon could not be demonstrated in the animal. Other interferon inducers were also effective in producing resistance but the complexity of responses to these inducers makes the precise mechanism unclear. Dr. John Barbaro discussed the mechanisms of histamine release in rabbit schistosomiasis. The essential requirements are leukocytes from an infected rabbit; with platelets from a normal or an immune rabbit, the same amount of histamine is released. Among the leukocytes, the lymphocytes were shown to be the important cell. Barbaro visualizes a two-step procedure; first the reaction of the sensitized lymphocytes with the antigen, and then the interaction of these lymphocytes with platelets which results in release of their histamine load. He has clearly shown that the antigen does indeed couple with the lymphocyte producing an "activated lymphocyte", and this occurs optimally at 37°, and that once activated, lymphocytes retain their effect on the platelet even though no longer viable.

Dr. E. J. L. Soulsby addressed himself to cell mediated immune responses, using Ascaris in the guinea pig. He demonstrated that there is inhibition of migration and of growth of the parasite in an immune animal. This cannot be reproduced by passive transfer of immunoglobulin but it can be transferred by lymphocytes. On challenge, focal areas of methyl-green-pyronine-straining lymph cells develop in the liver. These cells are also prominent in the lymph nodes. However in the lung, the response is very different with a plasma cell-eosinophil response in contrast to the lymphocyte-macrophage response seen in the liver. Similar changes occur in the normal animal but at a significantly slower pace. Thus it is possible for the same host, with the same parasite, and probably the same antigens, to have two apparently different immunopathological mechanisms. Dr. C.W. Kim discussed delayed hypersensitivity and lymphocyte transformation in Trichinalla spiralis. He was able to sensitize guinea pigs with a crude saline extract of T. spiralis larvae in complete adjuvant. On skin test 5 to 7 days later, a delayed hypersensitivity response occurred with induration and erythema; after 14 days the reaction was more of mixed delayed Arthus reaction with hemorrhage.

The delayed hypersensitivity was transferable to other animals by lymph node transfer. When cells from guinea pigs sensitized only once were transferred, delayed hypersensitivity but no secondary antibody response was present. When cells were transferred from animals repeatedly immunized, the transferred skin reaction is more like an Arthus reaction and the recipients do develop circulating antibody. On electron microscopy, lymphocytes from animals with repeated sensitization are reported to show much more prominent endoplasmic reticulum than those from animals with single immunization. After repeated immunization, systemic anaphylaxis was demonstrable; 50% or more died on challenge. In a cell culture system, lymph node cells from animals sensitized only once showed a low incidence of transformation into blast-like cells while the cells from animals after repeated sensitization very much more frequently transformed into blast-like cells when stimulated by antigen. Thus, the pattern of immunization influenced the response in the donor animal as well as in the recipient.

Dr. Warren summarized his work on delayed hypersensitivity and granuloma formation in schistosomiasis. He has demonstrated in mice that granuloma formation is the response to schistosome eggs, that granuloma are suppressed by immunosuppressive drugs, that their formation requires sensitization by prior exposure, that it is specific in that no reaction is noted in animals sensitized with *Ascaris* eggs and vice versa, that the reaction is specific for the specific species of schistosome involved, and that it is stage specific. The reaction is transferable by lymph node or spleen cells but not by serum. Rabbit anti-mouse lymphocyte serum virtually wipes out the granuloma formation around the eggs. Chronic X-ray radiation (30 roentgens 5 days a week for months) impaired antibody formation but had no suppressive effect on the development on schistosome granuloma. These properties are those of delayed hypersensitivity. Furthermore, he has been able to obtain a soluble substance from the schistosome egg which elicits or sensitizes mice to granuloma formation; when injected intraperitoneally without adjuvant, one microgram is sufficient to create hypersensitivity. This can be used for skin testing, and the granuloma formation proves to correlate excellently with the delayed skin test reaction. Thus this phenomenon, which is an adverse and nonprotective reaction of the body, fits all the criteria of delayed hypersensitivity.

Finally, Dr. R. T. Damian discussed antigens common to both host and parasite, the basis of a theory of the evolutionary adaptation of parasites to their hosts through the reduced foreignness of the parasite. After the possibility that host antigens might be physically carried over into the next stage in the life cycle has been excluded, the demonstrated presence of common antigens provides a device by which parasitism can persist without rejection.

The symposium was clearly a success; its best measure is found in the fact that almost as many pages of the transcript are required to present the discussion as were required for the presentation itself!

III. Commission-Sponsored Research

The annual progress reports of Commission-sponsored contracts are appended to this report. Highlights of progress and of the discussion at the meeting are presented here.

a. Dr. E. E. Baker (Immunologic Studies of Schistosomiasis). This project is in its terminal year. A variety of methods for fractionating the *Schistosoma mansoni* antigens were studied. Problems were encountered with aggregation of complexes; losses of material with each fractionation procedure were high resulting in little recoverable material. Although none of the methods tried have proven satisfactory, three fractions have been obtained which appear to contain only a single antigen; their immunogenic activity is under study.

b. Dr. G. Edsall (Factors Influencing the Pattern of Immune Response) has shown that a single dose of a maximally potent absorbed tetanus toxoid which caused no undue reactions induced a measurable antitoxin response in 50% of subjects within 14 days and a protective antitoxin level (i.e. greater than 0.01 antitoxin units per ml) was present of 16 of 18 subjects over one year later. As he had previously reported, the distribution of antitoxin titers at 28 days after the first injection of toxoid fell into a bimodal distribution. The higher titers were found in those under 18 years of age, with a suggestion that lower levels were found among the females. Of great practical importance is the observation that 2 inoculations, given at the proper interval, produced antitoxin levels equivalent or higher than those following 3 doses, regardless of the intervals in the latter group. When the interval between the first and second dose was more than 120 days apart, the geometric mean titers were twice as high as those following shorter intervals; a third dose contributed nothing. These studies promise that better immunity can be anticipated with a reduction on the number of inoculations to which the soldier is subjected.

c. Dr. H. N. Eisen (Antibody Structure and Function). These studies have had as their objective an understanding of the energetics involved in the antigen-antibody interaction and of the factors which determine the strength of the bond between the antigen and the antibody, a vital factor in determining the success of "neutralization". These studies depend on homogeneous antibody populations and for this Eisen has turned the immunoglobulin from mouse myeloma tumors; he uses a relative simple chemical as antigen. Two myelomas have been found to produce immunoglobulins which bind 2,4-dinitrophenyl (DNP) haptens selectively. Both of these antibodies have the characteristics of conventional anti-DNP antibodies, both of the purified 7S proteins have two identical and homogeneous binding sites per molecule of 150-155,000 molecular weight, each site situated in a Fab fragment of 55,000 molecular weight. The two isolated immunoglobulins had differences in aminoacid sequences but the patterns of cross reactivity to other antigens were similar to each other and to those of conventional antibodies. Two unusual cross reactions discovered with both myeloma proteins were also displayed by conventional antibodies, supporting other evidence that the binding sites of these myeloma proteins are authenic representatives of the sites on conventional antibodies. These studies have depended on the development of sophisticated methods for assaying antibodies which will facilitate proper interpretation of existing serological data.

d. Dr. K. F. Meyer (Studies on the Immunization of Man Against Plague) has extended his studies on the use of so-called avioulent living plague strains for immunization. Working with langurs, the EV76 (WR-S) strain which has been shown inadequately immunogenic in human subjects, did elicit good antibody response in langurs and this immunity against virulent challenge persisted for at least 16 months. The EV 51f strain, which was safe for guinea pigs and mice, caused vaccination fatalities in Ethiopian and Kenyan vervets, and in langurs; those animals which did survive vaccination were solidly protected. The Fraction-1-deficient, non-pigmented M23 strain, which is well tolerated by guinea pigs and rhesus monkeys, was lethal to 4 out of 4 langurs and must be classified as a "harmful" vaccine which only partially protects *Cerpithecus* monkeys. A transplant from the original live attenuated EV76, stored in Paris, was obtained; this produced local skin reactions and regional lymphadenopathy, positive blood cultures were obtained in some of these animals. On subsequent challenge, immunity was found; however, the question arises whether this vaccine which would be rated as "safe" for experimental animals is really harmless for man in view of the definite local and systemic side effects observed in these primates. The question can only be answered by careful studies on man. The effectiveness of Fraction-1 itself as the immunizing preparation was examined in langurs; it was given in adjuvant 65. Four out of six died after a cutaneous challenge, deaths were delayed, indicating some immunity.

e. *Dr. K. F. Meyers'* (Isolation and Characterization of Lymphogranuloma Venereum Agents) lymphogramuloma project has been activated for too short a time for a report; however, several new strains of lymphogramuloma virus have been isolated and are under study.

f. *Dr. A. G. Osler* (Studies on the Mechanisms of Hypersensitivity Phenomena) has continued his studies bearing on the allergic response. He has shown that leucocytes from immunized rabbits carry reagin-like antibodies and these cells release histamine on interreaction with specific antigen. He has shown that sensitized lymphocytes from nonimmunized rabbits (which do not contain histamine) react with antigen and destroy neighboring platelets which then release their contained histamine. Osler has also demonstrated that different classes of immunoglobulin affect the complement system differently. Gamma-I immunoglobulin isolated from guinea pig antisera mediate passive cutaneous anaphylaxis but fail to activate the complement system in conventional assays when antibody, complement, and antigen are added in that order. However, when preformed immune aggregates are added to the complement, fixation occurs. On the other hand, gamma-2 immunoglobulins effectively fix complement in either sequence of addition. Thus, there may be two distinct pathways that will have to be interrupted by different pharmacologic agents in order to prevent these mediators. Dr.

Osler's studies are providing the building blocks which will make possible control of the adverse reactions to immunization regimens.

g. Dr. J. W. Uhr (Antibody Formation & Immunity) has been concerned with the basic mechanisms in the synthesis and secretion of antibody and the regulation thereof. The feedback mechanism by which a serum antibody can suppress its own formation was described in his laboratory in 1960; this implies the possibility that a low level of serum antibody, insufficient to effect long term immunity, may interfere with continued immunization or successful reimmunization. Working with plasma cell tumors of mice and the incorporation of carbohydrate onto the immunoglobulin, he has shown that some glucosamine is incorporated into nascent polypeptide chains while they are on rough endoplasmic reticulum. The IgG then moves to the Golgi complex where more glucosamine, and galactose are incorporated into the IgG molecule. The immunoglobulin is then transported back over the cytoplasm of the cell and across the plasma membrane. Uhr studied the regulation of the antibody level by removing specific antibodies by exposing plasma to an immunoadsorbant and then returning the adsorbed plasma to the animal. The majority of the antibody activity was specifically removed without any effect on the level of other antibodies. One or two days later serum antibody level to this antigen began to rise and reached peak levels within 5 days; the levels of other antibodies did not change. This confirms the hypothesis that serum antibody does regulate the antibody response to metabolizeable antigens. Uhr has also been carrying out studies on the effect of streptococci on chimpanzees. Dispite a brisk anti-streptolysin-0 response in 12 chimpanzees after injection into tonsils and skin of Group A nephrotoxic beta-hemolytic strains, no definite evidence of sequellae has been obtained as yet.

h. Dr. W. F. Verwey (Responses of Humans to Cholera Vaccine Immunization) reported on his initial observations on the second group of volunteers immunized with varying schedules of cholera vaccine. Again a booster effect was not observed after the second dose but two doses produced higher geometric mean titers by eliminating poor responders. When the geometric mean titers of those receiving the two doses with a one week interval are compared to those with a four week interval, there is a significant difference in favor of the one week interval at 1 week, 2 weeks, and 6 weeks after the second dose. This advantage is shown to be derived primarily from the improved response of individuals who did not have preimmunization titers. The validity of these studies depends on the significance of the vibriocidal antibodies: in studies in Dacca the level of these antibodies correlated with protection. Recent studies there have shown that a monovalent Inaba vaccine was highly effective against Inaba cholera, while an Ogawa monovalent vaccine had no effect on the frequency of Inaba cholera disease. However, this vaccine did elicit anti-Inaba vibriocidal antibody. These observations bring out the importance again of the type-specific reactivity and cross reactivity in the cholera antigens. They indicate the vibriocidal titer may be a reliable measure, as is normally used, only with a bivalent vaccine. It should be recalled however, that a purified Ogawa antigen had been shown in 1964 to afford protection against Inaba disease to adults but not to children. Dr. Verwey's study group awaits the passage of time to answer the question whether a booster immune response can be expected after an adequate interval.

IV. Review of Areas of Concern of the Commission

a. Typhoid Fever

Dr. Hornick reported on the human volunteer studies underway at the University of Maryland. The effectiveness of oral typhoid vaccines has been restudied but again the challenge dose was higher than ID 25, the level at which discrimination was evident with parenteral vaccines. The oral vaccine did elicit a low level of antibody rise and doubling the dose increased the number of individuals showing a four fold rise in anti-0 anti-H and anti Vi antibodies. Of more interest was a study in which those who had developed clinical typhoid fever in previous experiments were rechallenged with 100,000 organisms; the incidence of clinical disease was not different from that among the control group. Dr. Mackiness suggested that the lack of resistance of those who had previously had experimental typhoid might be explained by the early and effective treatment which they received, just as in tuberculosis, where effective treatment renders the patient tuberculin negative and delayed hypersensitivity is completely lost after several months. Dr. Sanford brought out the fact that typhoid vaccine evolves primarily a gamma-M antibody response;

Dr. Edsall reminded the group that protection in the WHO sponsored field trials has lasted for seven years.

b. Cholera

Volunteer studies using cholera vibrios have been initiated by Dr. Hornick using the Inaba 569 B strain which has been shown to produce clinical disease in dogs when administered orally. With increasing challenges through 10^{10} vibrios, clinical typical cholera was not produced, although there was colonization (positive stools) in two out of four challenged with 10^8 vibrios, and one out of two to 10^9. However, when 10^{11} organisms were administered in 100 ml of beef bouillon, clinical disease ensued in one of two volunteers. When the challenge was given with 2 grams of sodium bicarbonate, colonization without disease occurred after a challenge with 10^4; with 10^6 vibrios typical clinical disease occurred approximately 36 hours after the challenge was given. These initial results demonstrate that the healthy American adult can be infected with cholera vibrios under appropriate conditions, and it will make possible evaluation of cholera vaccines in the laboratory.

c. Smallpox

(1) Eradication Program

Dr. Millar reviewed the world wide smallpox situation. In the Western Hemisphere disease exists only in Brazil where current systematic immunization programs promise eradication within the near future. An eradication program was started in Indonesia in March 1968 so that at present the disease is limited to central and west Java as well as some of the outlying areas in Sumatra and Celebes. In the Indo-Pakistan area, the number of cases was 40% lower than those reported last year. In Madras State, Dr. A. R. Rao has effectively eliminated endemic smallpox, demonstrating that eradication is possible.

In East Africa, smallpox persists in the Congo, Ethiopia and Sudan. Of great concern is the fact that there is no program in Ethiopia. In West Africa, the results are most encouraging. The National Communicable Disease Center's program has resulted in a strong sustained downward trend in cases, indicating success in the eradication program. In the discussion, questions of duration and protection were raised and Dr. Millar pointed out that 80% of smallpox cases occur in those who are totally unvaccinated.

(2) Methods of Vaccination

Dr. Benenson reported on the evaluation of the MonoVacc, which has nine plastic points mounted on a small square platform, which had been carried out at Fort Dix. Pressure with the MonoVacc was applied three times in the same site (27 insertion points), in comparison with 15 punctures with a bifurcated needle applied on the other arm at the same time. Four groups were vaccinated to permit comparison of "expert" vaccinators versus enlisted vaccinators who had had no previous experience with this technique, and to compare the performance of the MonoVacc using the supplied National Drug Vaccine, with the results when the standard vaccine on the supply line at Ft. Dix was used. There proved to be a greater difference in the percentage of major reactions based on the vaccinators (90.9 vs 80.5% for multiple puncture; 81.9 vs 75.5% for MonoVacc) than there was to the method of vaccination; there was no difference in MonoVacc results attributable to the vaccine used (79.5 vs 78.4%). Accordingly from a technical point of view the use of this device for smallpox vaccination was approved.

d. Adverse reaction to immunization

Four days after the smallpox study above was performed, in which both arms were vaccinated, TAB vaccine and Td toxoids were administered to one of those companies. Within the next several hours 88% had reported on sick call and 46% of the company was hospitalized. Relating hospitalization to the type of vaccination reaction present on the seventh day, 49% who had had a major reaction had been hospitalized in contrast with 13% of those who had had only an equivocal reaction. (Table A). To confirm the association with the major reaction, twenty-five men with a pustular reaction on the

Table A

Vaccination Reactions versus Hospitalization after Td & TAB immunization

Primary	24/37 *	65%	Equivocal	2/14	
S+	45/105	43%	Not vaccinated	1/9	
Total Major	69/142	49%	Total	3/23	13%

*Number hospitalized with chills and fever on day 4 over number with primary type vaccination reaction on day 7.

seventh day after smallpox vaccination were given a dose of Td into that arm. A comparable group was given TAB, and another 25 men were given Td and TAB both into the same arm. As a control, 110 men with pustular reactions were given both Td and TAB into the other arm. Within several hours, 16%, 28%, 24% and 4% of these groups respectively developed chills and fever for which they sought medical care, confirming the hypothesis that injection into the area of vaccinal reaction was responsible for the reaction. It is interesting that reaction rate following the injection of Td was comparable to that following the parogenic TAB. That this phenomenon had not been previously observed was puzzling; suggestions included the possibility that the regional lymph nodes had lost their filtration capability or possibly that the individuals were selected by increased pain when the antigen was injected into a previously inflamed area.

e. Plague

Dr. Cavanaugh reviewed the plague experience of the U.S. military personnel in Vietnam. Studies carried out on rats have shown that they are comparably infected with murine typhus and with plague. However, among military personnel, 20% of FUO's have been shown to be due to murine typhus but only eight cases of plague have been diagnosed since 1964. Over the same interval, many thousands of cases have occurred among Vietnamese civilians. American personnel are billeted within the civilian areas where rats are prevalent and where plague is common among the civilian population, even though forty percent of this population has been vaccinated with the Saigon attenuated strain each year. Evidence that our personnel are actually exposed to plague is found in a study of 64 individuals who spent 6 months in Vietnam (without receiving a booster dose of vaccine during this interval); 55 (or 86%) showed a significant rise in titer during their sojourn in Vietnam. From this it is presumed that adequate exposure is occurring and that protection afforded by the inactivated plague vaccine in standard military use is indeed effective.

f. Yellow Fever

Dr. Wagner reported on the status of the trials of the new avian leucosis-free line of yellow fever vaccine. Four lots of the new vaccine will be compared at Fort Ord with four lots of the old vaccine for reactions and antigenicity within the next few months. The new vaccine has presented no evidence of any difference in neurovirulence for monkeys, antigenicity, or any other known property.

g. Mumps

Routine immunization against mumps is not recommended for military personnel; its use is limited to outbreaks of mumps. In view of the effectiveness of the new attenuated vaccine now available, this policy was questioned and the military experience with mumps requested. Army experience from 1958 through June 1969 revealed a gradual decline from a rate of 1.59 per 1,000 in 1958 (1.50 within the U.S. and 1.72 overseas) to 0.35 in 1969 (0.41 in U.S., 0.28 overseas). The actual number of cases was 1361 in 1958 and 557 for 1968. The smooth trend was broken in 1960 by the development of 2144 cases, with rates of 2.57 (2.28 in U.S and 3.00 overseas). Since mumps poses no practical problem at this time, the present policy does not require change.

h. TB Med 114 - Immunization

The final draft of TB Med 114 was submitted to the Surgeons General in late June; the triservice final version was reviewed in December, 1969, and several corrections noted. The corrected version is expected to be in the hands of the printers by the end of January, 1970.

V. Reports of New Work and New Investigators

a. Dr. W. A. Altemeir (The Efficiency of IgM antibodies to the 0 antigen of *S. typhosa*) is preparing to follow up his earlier observation that typhoid vaccine elicits a specific IgM antibody response with peak levels usually after 7 to 10 days. He plans to determine the efficiency of IgM antibody, defining this as the bacteriocidal activity per unit of specific antibody, with the objective of determining the factors (immunization regimens and natural infection) which results in the best antibody response based on quality as well as amount.

b. Dr. E. E. Baker (A Chemical Biological Study of the Antigen of *Pasteurella pestis*) reviewed the background for the preparation of Fraction 1, originally isolated by him in 1952. Since this is the essential reactant in serological tests for plague, a highly purified and standardized Fraction 1 is needed for interlaboratory comparability of results. With present analytic methods, it becomes clear that several antigens are present in the earlier preparations. Purification involves the problems that there is a carbohydrate containing components (Fraction 1A) and one free of carbohydrate (Fraction 1B), and media ingredients, such as agar, further complicate the objective of separation without significant processing losses.

c. Dr. N. P. DiLuzio (Role of the Reticuloendothelial System and Opsonins in Malaria and Other Parasitological Infestations) has had a great deal of experience with the reticuloendothelial system. He has been using the neutral polysacharide glucan to potentiate reticuloendothelial function, which it does by producing proliferating macrophages. Contrary wise, profound and prolonged impairment of function can be induced by the use of methyl palmitate. He plans to investigate the effect of malarial and typanisomal infections on host phiagocytic functions, the effect by variations of reticuloendothelial function on the parasitic infections, and related phenomena.

d. Dr. B. B. Levine (Classes and Properties of Human Antibodies) has been studying the control of reagin synthesis in mice, finding that only some mouse strains are good reagin producers. He has been studying the effect of varying dose and type of antigen on the reagin production. He also has been working with tests in man for reagins to penicillins; he tests for these by using benzylpenicilloyl polylysine and a minor determinant mixture as skin test antigens using a wheal and flare reaction within 10 minutes as the criterion for positives. Of 218 patients with a history of penicillin allergy, 85% gave negative skin tests and were treated with penicillin without desensitization and without immediate reaction. Eight of eleven with postive skin tests who were treated after desensitization developed accelerated or immediate urticasial reactions. Unfortunately, these test antigens are not available commercially. He wishes to continue his studies on penicillin hypersensitivity in man, and to continue studies on factors controlling reagin synthesis and the initiation of anaphylactic reactions.

e. Dr. N. R. Rose (Cellular & Humoral Immunity in Parasitic Infections) proposes to develop a mechanism for measuring cell-mediated immunological reactions which are analogous for the cellular immunologist to the precipitation and complement fixation techniques for the serologist. His initial approach is based on the observation that populations of lymphocytes can kill lymphocytes from another species against which the original donor animal has been immunized. The killing effect is detected by the reduction in the number of target cells producing plaques in the Jerne technique. He proposes to approach the problem, using toxoplasma as the target cell, initially planning to label these with chromium-51 or other suitable label so that the release of intracellular constituents can be quantitated.

f. Dr. W. L. Ryan (Macrophage in the Immune Response) has been studying the macrophage, particularly the transport of proteins into the cell. He has found that there is no greater uptake of antigen by the macrophages from an immune animal than from a nonimmune. However, if the small amount of antiserum is present, then there is an increased uptake of antigen. He has also been following the degradation of antigens by the cell, using lysosomal cathepsins in vitro. He wishes to extend these observations to understanding the metabolic route leading to an immunogen within the macrophage.

g. Dr. A. M. Silverstein (Fetal Response to Immunization) reported on a program that has been underway for 7 or 8 years, studying the development of immunological competence in the fetus of the sheep, which can be manipulated and replaced in the uterus without causing abortion. He has found that recognition of different antigens begins at different times but when immunological competence for an antigen does appear, there is no evidence that the response to that particular antigen is "immature" in any way. Thymectomy can be performed at 48 days gestation (the gestation period of the sheep is 150 days) with no direct effect on the immunological maturation of the fetal lamb; there is a lymphopenia but its ability to reject graft or its ability to make antibody are unimpaired. Thymectomized fetuses have gone to term and one prenatally thymectomized sheep is now 4 years old with a chronic lymphopenia but immunologically competent in every respect. The part played by gut-associated lymphoid tissue was investigated by ablation of the entire intestinal tract prior to the time when the first lymphocyte arrives in the lamina propria; this had no effect on the competence of the animal. Contrarywise, putting an antigen into the intestinal tract stimulated precocious follicular activity and lymphoid maturation. Some of these studies have been replicated and confirmed in fetal monkeys. This approach provides the opportunity to define the relationships between antigen properties and the state of development of the lymphoid tissues, as well as to explore the requirements for immune tolerance.

VI. Accomplishments during the year

Significant progress has been made in several areas of concern to the Commission on Immunization.

a. Steady progress has been made in the definition of immunity to plague. Earlier high hopes for a live attenuated vaccine have been dampened by the reactions seen in certain species of sub-human primates; these make it unlikely that any present attenuated live vaccine could be used for immunization of population groups without hazard to some members of the population. On the other hand, experience of U.S. personnel serving in plague-infected areas is for the first time giving us assurance that our current immunization program, developed empirically, is affording a high degree of protection.

b. Data developed during this year on immunization against tetanus offer great promise that the toxoid and the immunization regimen can both be improved so that more effective protection can be achieved with a reduction in the number of doses required, minimizing the number of adverse reactions and the loss in training time.

c. In the field of cholera immunization, studies under way are suggesting that there may be a significant advantage in using a shorter, rather than a longer, interval between doses.

d. In the area of smallpox vaccination, a commercially available device for performing the vaccination has been evaluated and shown to be effective when properly used.

e. Steady progress is being made in the understanding of the mechanisms, cellular, pharmacological and immunologic, which are involved in allergic reactions both those occurring naturally and those that follow immunization procedures.

f. Field studies carried out under Commission auspices have defined shot-line conditions which will minimize the loss of training time due to adverse reactions.

g. Commission sponsored studies during the year have provided better understanding of the structure and function of the immunoglobulins, and the place and control of their synthesis, advancing us toward our ultimate objective of being able to manipulate the antibody response for maximal protection against present or future infecting agents and minimal reaction.

h. Under Commission auspices, TB Med 114 Immunization has been completely revised and brought up to date.

VII. A final report by Dr. Ralph S. Wedgwood on "Normal Human Serum" is enclosed. This project was completed on 31 July, 1967. The Director wishes to thank the members of the Commission for their cooperation and support.

Respectfully submitted,

Abram S. Benenson, M.D.
Director
Commission on Immunization

ASB/mb

AD______________________

Immunologic Studies of Schistosomiasis (U)

ANNUAL PROGRESS REPORT

by

Edgar E. Baker, Ph.D.

August 1969

(for the period 1 July 1968 to 31 May 1969)

U.S. Army Medical Research and Development Command

Washington, D. C. 20315

Annual Report to the Commission on Immunization
of the Armed Forces Epidemiological Board

Contract No. DA-49-193-MD-2883

Boston University School of Medicine

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Foreword

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

Last year it became evident that at least some of the difficulty in handling extracts of adult Schistosoma mansoni was due to losses resulting from denaturation. As a result of studies reported last year, better methods of extraction, storage, and concentration were developed. It also became apparent that gel filtration was not practical. Although preliminary ultracentrifuge studies suggested molecular weights of about 100,000 for most of the antigens, there was considerable retention even by sepharose which excludes molecules below a molecular weight of 200,000. The work this year has been largely concerned with the use of ion exchangers, DEAE cellulose, DEAE sephadex and CM sephadex. As in the past, lyophilized adult S. mansoni were supplied by Dr. Elvio Sadun, Walter Reed Institute of Research.

Ultracentrifugation in a Sucrose Gradient: Preliminary experiments begun last year were extended in an effort to separate worm antigens by centrifugation in a sucrose gradient. The material was centrifuged in 30 ml tubes at 98,000 X G in a Spinco Model L with SW 25 rotor for 18 hours. In separate experiments, gradients of 20% to 5% sucrose and 15% to 3% sucrose were used, and in both cases, the antigens always entered the gradient in a group as if they were complexed and no separation was effected.

Treatment of extracts with Sodium Lauryl Sulfate: The failure to separate antigens by centrifugation in a sucrose gradient possibly might have been due to interaction of individual proteins. It was thought that it might be possible to disaggregate such complexes by treatment with sodium lauryl sulfate. 0.1% and 0.01% sodium lauryl sulfate was used to treat saline extracts of worms or was used during extraction of adult worms. When tested by gel diffusion, there was no evidence of dissociation of antigens, The additional problem of the inability to remove the detergent from the treated antigen preparation forced us to discard this method of treatment of antigen preparations.

Fractionation of antigens by chromatography on DEAE cellulose: As was reported previously, gel filtration on Sephadex and Sepharose gave poor separation of antigens from crude extracts. It was felt that ion exchange chromatography might be a better means of separating antigens.

In order to determine optimum conditions, a series of small batch type experiments were conducted. Since extracts buffered with Tes (N-tris (hydroxy-methyl) methyl-2-amino-ethane sulfonic acid) at pH 7.2 were most stable under a variety of conditions, this buffer, usually in a concentration of 0.01 M, was used in all experiments. Small amounts (usually 1 mg of protein) of antigenic material were mixed for 1 hour in the cold with varying amounts of DEAE cellulose or CM cellulose equilibriated in 0.01 M Tes pH 7.2. This was done to determine both the ratio of gel to sample which would give the greatest adsorption and which gel would be most useful. After washing with buffer, all samples were eluted with 0.5 M NaCl in 0.01 M Tes pH 7.2 for ½ hour with occasional mixing. Both the supernatent and the eluate were tested serologically by gel diffusion for removal and recovery of antigens. DEAE was tested in ratios to antigen sample of 100:1, 40:1, 20:1, 10:1, and 5:1. When less than a 40:1 ratio was used, not all of the antigens were adsorbed. With 40:1 or 100:1 ratios, 4 of the 5 antigens present in the extracts were adsorbed. However when the 100:1 ratio was used, the antigens would not be effectively eluted. CM cellulose did not adsorb any antigenic material.

The data from the above experiment indicated that DEAE cellulose chromatography might be an effective means of separating schistome antigens. A saline extract of adult worms, containing at least 5 antigens by gel diffusion tests was put on a DEAE column with a ratio of gel to sample of slightly greater than 40:1. The material was eluted with a gradient from 0.01 M Tes pH 7.2 up to 0.7 M NaCl in 0.01 Tes pH 7.2. All antigens were adsorbed but essentially none could be detected in the eluate. Further washing of the column with 1 M NaCl buffered with glycine pH 9.0 did not elute any of the antigens. Because of the difficulty in elution at a ratio of 40:1 and the failure of all antigens to adsorb at lower ratios it was decided to test DEAE Sephadex.

Fractionation of antigens by chromatography on DEAE Sephadex A-50: Again batch experiments were done prior to the use of columns. These experiments were carried out as described above for DEAE cellulose using ratios of gel to antigen samples of 40:1, 20:1, and 10:1. Ratios of gel to samples of 10:1 and 20:1 adsorbed 4 of the 5 antigens as in the case of DEAE cellulose.

A preliminary experiment using a very small column of DEAE Sephadex A-50 showed that material could be eluted in contrast to the experiment with DEAE cellulose. Further, it was found that crude extract could be separated into three fractions.

It was felt that perhaps even better antigen separation might be effected equilibrating the gel and dissolving the sample in a buffer containing NaCl and thus decreasing interaction among the proteins present. Batch experiments showed that the antigens would adsorb to Sephadex A-50 satisfactorily in either 0.05 or 0.1 M NaCl. Therefore, it was decided to fractionate extracts in 0.1 M NaCl in 0.01 Tes buffer.

Three identical columns of DEAE Sephadex were used to fractionate three extracts prepared in exactly the same way in an effort to determine reproducability. A typical experiment was as follows: 75 mg of defatted worms were suspended in 15 ml of 0.01 M Tes pH 7.2 and mixed in the cold for 2 hours. The suspension was centrifuged at 23,000 X G for one hour and the cleared extract removed. The worms were resuspended and re-extracted twice more. The pooled extracts were concentrated by lyophilization, redissolved in 1.2 ml of water and dialyzed for two hours in the cold against 0.1 M NaCl. The material was cleared of any insoluble substance by centrifugation at 10,000 X G for 1 hour and the entire sample put on the column except for a small amount reserved for serologic testing. The sample put on the column contained 21 mg protein per ml and at least 5 antigens by gel diffusion. Serologic activity of at least one antigen was still evident at a dilution 1:1024. The column contained approximately 500 mg DEAE Sephadex and measured 17x0.6 cm. The eluant was pumped through the column at a rate of 5 ml per hour. 1 ml samples were collected. After the void volume was collected, a gradient was established by the addition of 0.7 M NaCl to a 75 ml mixing bottle. A total of 125 samples were collected from the column.

Protein was measured in each sample collected from each column by the method of Groves, Davis and Sells (Analytical Biochem. 22, 195, 1968). Each sample was tested by gel diffusion using rabbit antiserum to the whole worm. On the basis of serologic tests, the 1 ml samples could be grouped to give three fractions. The first group represented the material excluded from the DEAE Sephadex. It contained at least 2 antigens which were different from any found in the other groups. The second group eluted between 0.2 M and 0.4 M NaCl comprised a greater number of samples from the column and contained at least 3 antigens. The third group eluted between 0.5 M and 0.6 M NaCl, contained one antigen different from any found in other groups. The individual samples in this experiment were not pooled at this time but stored frozen.

The above experiment was repeated twice. Similar results were obtained from each column. Corresponding fractions of each column were examined by gel diffusion and the antigens in each corresponding fraction were found to be identical, therefore they were pooled and labeled Fraction 1 (2.5 ml), 2 (40 ml) and 3 (60 ml). Each of the fractions was desalted on a column of Sephadex g-25. The portion of eluate containing antigen was lyophilized.. This method of removing salts was unsatisfactory because marked loss of material occurred on the column and the method has not been used subsequently. Each fraction was redissolved in 1 ml of 0.01 M Tes, pH 7.2, tested by immunoelectrophoresis and titration in gel diffusion plates. In addition, 0.1 ml of each of the redissolved fractions was emulsified in complete Freund's adjuvant and used to immunize rabbits. Each rabbit then received a booster injection of 0.25 ml antigen in the same manner two months later. The resulting antisera were examined by gel diffusion.

Fraction 1 contained at least two antigens by immunoelectrophoresis and both of these precipitated with antiserum against the whole worm when tested in gel diffusion plates at dilution of 1:8 but did not precipitate at higher dilutions. Rabbits immunized with Fraction 1 produced antiserum which contained at least four antibodies when tested (gel diffusion) against Fraction 1 (homologous), one antibody detectable against Fraction 2 and two antibodies reacting against Fraction 3.

Fraction 2 contained at least 3 antigens by immunoelectrophoresis and one antigen which reacted with infected human serum. Titration of precipitating antigen by gel diffusion revealed that the material could be diluted to 1:32 and still react with rabbit antiserum against the whole worm. The rabbit antiserum against Fraction 2 contained at least 5 antibodies which precipitated with Fraction 2 (homologous), 1 antibody precipitated with Fraction 1 and two which reacted with Fraction 3.

Fraction 3 contained a single antigen by imminoelectrophoresis and this antigen diluted 1:64 could still be detected by gel diffusion. The rabbit immunized with Fraction 3, however, produced two antibodies as tested by gel diffusion with Fraction 3 (homologous) and one and two which reacted with Fraction 1 and 2 respectively.

These results indicate that the immunogenic activity of an antigen preparation is a much more sensitive method for detecting impurities than is serologic testing by gel diffusion. It is also evident that only very small amounts of schistosome antigens are necessary to stimulate antibody production.

Fractionation of Antigens by Combined DEAE Sephadex Chromatography and Pevikon Block Electrophoresis. In view of the partial success in separating antigens by means of DEAE Sephadex Chromatography, another experiment was designed to decrease losses through handling, by chromatographing a large amount of crude extract and then using other methods for further purification of the factions obtained. It had been noted in the past that antigens in the crude worm extracts could be separated by acrylamide gel electrophoresis as well as in agar gel electrophoresis. It was thought that further purification of antigens in the fractions might be effected by Pevikon (Mercer Chem. Co., N.Y., N.Y.) block electrophoresis. Pevikon was chosen rather than starch because it is said to be less likely to adsorb proteins and it is somewhat easier to handle.

450 mg of defatted worms were extracted with 0.01 M Tes, pH 7.2 and the extract lyophilized. The extract was redissolved in 3 ml of water and dialyzed against 0.01 M Tes in 0.1 M NaCl. After dialysis, the material had a volume of 4.5 ml and the protein content was 30.4 mg/ml. By acrylamide gel electrophoresis, there were 9 components which stained with Aniline blue black (for protein) and 7 zones of precipitate when tested by immunoelectrophoresis and by gel diffusion tests (serologic activity titrated to 1:512) using rabbit antiserum to whole worms. The results of serologic titrations of this and subsequent fractions obtained are tabulated in Table I. 4.1 ml of the original extract was put on a column containing 2.5 grams of DEAE Sephadex A-50 which had been equilibrated with 0.01 M Tes on 0.1 M NaCl. The column measured 29 cm high by 1.7 cm in diameter. After 30 ml was collected from the column, a gradient was established by the addition of 0.7 M NaCl to the original buffer. The flow rate was 6.4 ml per hour and 3.2 ml samples were collected. Samples 13-20 comprised Fraction 1 (5.6 mg total protein before concentration) in which the antigens present were identical to those of Fraction 1 in the previous experiment. Samples 21-38 made up Fraction 2 (30.6 mg total protein before concentration). The pooled samples contained at least 3 antigens detected by gel diffusion. Fraction 3 (5.4 mg protein before concentration) was made up of samples 39-59 and had only a single serologically active substance. In view of the considerable loss of antigens by desalting with Sephadex G-25, it was not used in this experiment. Previous experiments had shown that extracts could be lyophilized with little or no loss and could be dialyzed against dilute buffers for short periods with no loss. Therefore, Fractions 1 and 3 were concentrated by lyophilization, dissolved in a small amount of water, dialyzed in the cold against 0.01 M Tes buffer for 4 hours with stirring and frequent changes of buffer. Using the same conditions, it was found that the conductivity of 0.5 M NaCl dialyzed against distilled water was reduced to a level only slightly above that of the distilled water

used. Fractions 1 and 3 were each lyophilized and then dissolved in 3 ml of water. Fraction 1 contained 2.4 mg total protein, 4 protein staining components by acrylamide gel disc electrophoresis, 2 serologically active components by immunoelectrophoresis and gel diffusion of which one antigen precipitated at a dilution of 1:64 in gel diffusion plates. Fraction 3 contained 4.3 mg total protein, 7 components by acrylamide gel disc electrophoresis and, in this concentrated form, 4 antigens (1 titrated to 1:128) could be detected serologically by gel diffusion.

Considerable loss occured during concentration of Fractions 1 and 3. The double lyophilization together with dialysis probably was responsible for the loss. Therefore, it was decided to hold Fraction 2 in the dry state without dialysis. Experiments were conducted which showed that negative pressure dialysis with the Schleicher and Schuell collodion bag apparatus (Keene, New Hampshire) could be used with minimal loss.

About 2.5 ml of Fraction 1 was placed in a collodion bag and by negative pressure dialysis against 0.01 M tris pH 8.6 was concentrated to 165 ul in 3½ hours. A 1:40 dilution of the concentrated fraction contained at least two antigens by gel diffusion tests, 140 ul of the concentrated sample containing 1.12 mg of protein was mixed with dry Pevikon and introduced into a Pevikon block 45 x 2 x 1 cm. equilibrated with 0.1 M tris pH 8.6. Electrophoresis was conducted at 4 C for 22 hours at 15 milliamp. current. The entire block was cut lengthwise into 1cm segments and each segment was suspended in 1.5 ml cold saline. The segments were numbered 1 to 45 consecutively from the cathode to the anode end of the block. Each segment was mixed, centrifuged to remove the Pevikon, and the supernatant tested by gel diffusion. Optical density readings for protein analysis was hindered by buffer interference. The Pevikon from each segment was washed repeatedly (six times) until no more serologic activity could be detected in the supernatants. Segments 14 through 21 contained a single antigen by gel diffusion tests. These segments were pooled and concentrated by negative pressure dialysis to 1 ml. In this material, the single antigen could still be detected when the material was diluted to 1:32. The fraction contained 0.18 mg total protein by the method of Lowry, Rosebrough, Farr and Randall (J. Biol. Chem. 193:265, 1952). 0.25 ml of the concentrated material was emulsified in Freund's complete adjuvant and administered to one rabbit.

Fraction 3 was handled in the same way as Fraction 1. 2.7 ml of Fraction 3 was concentrated to 230 ul containing 2.67 mg protein and four antigens detectable by gel diffusion. This fraction was put on a Pevikon block and subjected to electrophoresis as described above. After eluting the 1 cm segments of Pevikon, one antigen was detected in the eluate of segments 26-35. The eluate and washings from the Pevikon from segments 26-35 were pooled and concentrated to 1 ml. In this material, the antigen could still be detected by gel diffusion when titrated to a dilution of 1:256. The fraction contained 0.9 mg total protein as determined by the method of Lowry et al. 0.25 ml of the purified material was used to immunize a rabbit as was Fraction 1.

Although there appeared to be a loss of material by electrophoresis on Pevikon, the fractionated antigens appeared to be single entities in Fractions 1 and 3. Tests for purity are still in progress. Antibodies from rabbits being immunized with these fractions will be studied.

Fraction 2 appeared somewhat more complex. Since difficulty was encountered by buffer readings alone in optical density scans, Fraction 2 was reconstituted from the lyophilized state in about 5 ml of 0.01 M veronal-acetate (Michaelis) buffer, pH 8.6 and dialized against this buffer by negative pressure dialysis to a concentration of 1.4 ml. This material containing 6 antigens by gel diffusion could be analyzed for protein by O.D. at 280 mu and 1.2 ml contained 30.6 mg total protein which was subjected to Pevikon block electrophoresis. Gel diffusion analyses of the eluates and washes from segments of Pevikon showed some separation. Segments 18 through 24 contained three antigens. Segments 25 through 30 contained two antigens one of which was different from any found in preceeding segments. Therefore, two groups were made from eluates and washes of Pevikon segments from Fraction 2. Group 1 was a pool of material eluted from Segments 18-24 and Group 2 was a pool from Segments 25-30. Each was concentrated to a final volume of 1 ml by negative pressure dialysis. After concentration, Group 1 contained a total of 0.613 mg protein by the method of Lowry et al. and four antigens could be detected in the concentrate. However, serologic activity could be detected only to a dilution of 1:8. Group 2 concentrate contained 2.77 mg total protein by the method of Lowry et al and two antigens were detected in the concentrated material. When the material was diluted 1:16, serologic activity of one of the antigens was demonstrable but not in higher dilutions.

Although the use of Pevikon Block Electrophoresis appeared useful because there was some antigenic separation, recovery of material put on the Pevikon block was very low. The Pevikon used in electrophoresis of Fraction 2 was repeatedly washed with buffer and then with 0.5 M NaCl. However no additional antigenic substance was eluted. Even a subsequent pH change by washing the Pevikon in glycine-NaOH buffer at pH 9.0 did not elute any antigenic material or measurable protein.

Discussion

None of the methods outlined above for the fractionation of adult schistosomes has proven entirely satisfactory. Fractionation and subsequent concentration led to marked loss of substance and concomitant loss of serologic activity. However, three fractions have been obtained which appear to contain only a single antigen. The immunogenic activity of these is under study.

TABLE I

ANTIGEN SEPARATION BY DEAE SEPHADEX A - 50 AND PEVIKON BLOCK ELECTROPHORESIS

	Total Volume	Total* Protein	Minimal Number of Antigens ** detected at concentration of ug protein per ml													
	ml	mg	30,000	6,000	3,000	1,500	750	375	188	94	47	24	12	6	3	2
Crude Extract	4.5	137.0	7		6	6	5	4	3	3	2	0				
4.1 ml Crude Extract to DEAE Sephadex A-50 Column																
Fraction 1 a#	3.0	2.4					2	2	2	2	1	1	1	0		
2.1 ml Fraction 1 a concentrated to 0.14 ml to Pevikon Block Electrophoresis																
Fraction 1 b##	1.0	0.18							1	1	1	1	1	1	0	
Fraction 2 a#	5.0	30.6		6	6	5	4	3	3	3	2	0				
4.3 ml Fraction 2 a concentrated to 1.2 ml to Pevikon Block Electrophoresis																
Fraction 2 b Group 1 ##	1.0	0.61					4	3	2	1	0					
Fraction 2 b Group 2 ##	1.0	2.16			2	1	1	1	1	0						
Fraction 3 a#	3.0	4.35				4	4	3	2	1	1	1	1			
2.7 ml Fraction 3a concentrated to 0.23 ml to Pevikon Block Electrophoresis																
Fraction 3 b##	1.0	0.9					1	1	1	1	1	1	1	1	1	0

*Determined by the method of Lowry et al. (J. Biol. Chem. 193:265, 1951)
**Determined by gel diffusion tests according to Crowle (*Immunodiffusion*, 1961)
#Obtained from DEAE Sephadex A-50 Column
##Obtained from Pevikon Block Electrophoresis

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13. ABSTRACT

Several methods of fractionation of antigens of Schistosoma mansoni were studied. Extracts were prepared from delipidized adult worms by grinding the organisms in Tes buffer, pH 7.2 in the cold. Such extracts contained 5 to 7 antigens depending upon the concentration tested. It was found that antigens were not separable by ultracentrifugation in sucrose gradients. Antigens migrated in a group suggesting some complexing of protein molecules. In an effort to disaggregate any complexes, sodium lauryl sulfate was used. This was not successful because of inability to remove detergent. Antigens were adsorbed on DEAE cellulose columns but it was not possible to elute antigens even with the use of strong salt solutions or by a pH shift in the eluting buffer. It was thought that Sephadex ion exchangers might effect separation. Crude extracts were fractionated on columns of DEAE Sephadex A-50. Samples from each column were pooled in three fractions yielding 2, 3, and 1 antigens respectively. These fractions each were used to immunize rabbits but none of the antisera obtained reacted solely with its homologous antigen. There was some cross reaction with the other fractions. Another larger amount of crude extract was chromatographed on a column of DEAE Sephadex A-50 in order to obtain partially purified antigens that could be further fractionated by electrophoresis. Three fractions were obtained as described above and each was subjected to Pevikon Block Electrophoresis. There appeared to be some further antigen purification and these preparations were used to immunuze rabbits. However, losses of material through each fractionation procedure were quite high resulting in very little recoverable material.

DD FORM 1 NOV 65 1473 REPLACES DD FORM 1473, 1 JAN 64, WHICH IS OBSOLETE FOR ARMY USE.

14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	W
Schistosoma mansoni						
antigens						
fractionation						

AD____________________

FACTORS INFLUENCING THE PATTERN OF THE IMMUNE RESPONSE (U)

ANNUAL PROGRESS REPORT

by

Geoffrey Edsall, M.D.

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SUMMARY

A single dose of a maximally potent adsorbed tetanus toxoid, prepared in conformity to U. S. Public Health Service Minimum Requirements and causing no undue reactions, induced a measurable antitoxin level in 50% of the subjects within 14 days, and a protective antitoxin level (i.e. >0.01 antitoxin unit/ml) that was still present in 16 of 18 subjects over 1 year later.

The 28-day and 1-year antitoxin levels in these subjects showed a striking inverse relationship to age (range 14 to 39 years). The 4- to 5-year residual antitoxin levels after standard tetanus immunization in another group showed a similar (though less striking) inverse relationship.

Two doses of tetanus toxoid, given more than 90 days apart, yielded a residual antitoxin titer, 4-5 years later, approximately equal to that following 3 doses of toxoid, irrespective of the intervals in the latter group. Analysis of residual antitoxin levels in children showed a clear inverse relationship between number of injections and slope of the antitoxin fall-off rate with time.

Characterization of the components of tetanus toxin and toxoid -- shown to be complex by recent work of others besides ourselves -- has been facilitated by the finding of a culture technique giving much higher yields of the smaller antigen molecule associated with tetanolysin and probably with Feigen's "peripherally acting" toxin.

The presence of soluble endotoxin in typhoid vaccine was implicated as possibly distorting potency tests of the vaccine.

FOREWORD

This report summarizes work performed under Contract DA-49-193-MD-2144 with the Office of The Surgeon General, United States Army, and sponsored by the Commission on Immunization, Armed Forces Epidemiological Board. The contract with Harvard University is administered through the Harvard School of Public Health. The work is actually carried out at the State Laboratory Institute of the Massachusetts Department of Public Health in Forest Hills, a few miles from the Harvard Medical-Public Health area. The work has been made possible only through the very extensive contributions not only of individuals on the State Laboratory Institute payroll whose extra time and efforts are also compensated, in a small degree, through the Contract, but through the regular and essential contributions of more than 30 members of the staff of the State Laboratory Institute, without whose efforts in maintaining the regular services of all types in the Institute, the operation of the contract in question would be impossible.

Valuable and gratifying collaboration with colleagues outside the Institute has again included:

Dr. Kenneth W. Newell, WHO, Geneva
Prof. Paul Beaver, Dr. Robert MacLennan, Dr.. Mark Belsey, and the ICMRT staffs at Tulane University and the Universidad del Valle
Dr. Thomas C. Peebles, Weston, Massachusetts.

In conducting the research described in this report, the investigators have adhered to the "Guide for Laboratory Animal Facilities and Care" as established by the National Academy of Sciences-National Research Council.

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Factors Influencing the Pattern of the Immune Response

Geoffrey Edsall, M.D., Principal Investigator

As it has been from the beginning of the contract, the essential focus of the studies carried out during the year being reported was on the general problem specified in the original application for support under this contract which said

> "The background of the proposed investigation derives from the still extensive gaps in present knowledge of the factors determining the rate, extent, duration and reactivation of the immune response, in man, to the various antigens currently used or likely to be used for active immunization against infectious disease."

This concept has been embodied in the title of the project, "Factors Influencing the Pattern of the Immune Response". Obviously, however, such a broad concept must have defined limits in any one contract. The specific areas to which the studies have been directed have been the following:

a. Factors determining the scope and rate of the early primary response ("EPIR") (i.e. the response following a single dose of the antigen) with the object of either enhancement, acceleration, or both, of this response. Tetanus toxoid has been used as a model, first in animals to develop guidelines, and then in man for definitive testing. The study has been directed primarily toward the modification of the antigen preparation in such a way that the immune response would be accelerated or enhanced. This has included studies of the effect of aluminum salts, endotoxins and "endotoxoids", water-in-oil adjuvants, Poly-A-Poly-U complexes, antigen aggregation, and a few other miscellaneous factors, all of which are known to play a role in the degree of the primary immune response obtained. The working assumption of this portion of the study has been that, with the identification and use of appropriate enhancing mechanisms, acceptable for use in man, the primary immune response could be enhanced far beyond what has ordinarily been assumed to be possible. The work reported in the last two years of this study has amply confirmed the correctness of this assumption (see publications #5, 8, 10, 11, 12, 16 and papers in preparation).

However, it has also brought to light previously ignored or unnoticed factors affecting the immune response -- factors concerning the physiological status of the host. Attention has been given to these in the analysis of the data obtained to date and in planning of future studies; however, it is recognized that such areas were not forecast in the original or subsequent statement of the scope of the study, and the appropriateness of a study of host factors in the primary immune response may require review.

Since the existing methods of measuring the immune response to tetanus toxoid are sensitive only to the level of about 10^{-11} moles (approx. 10^{-13} molecules) of 7S antibodies to the toxoid, and since the EPIR has not yet been successfully and definitively detected earlier than about 4 days in the mouse and 14 days in man, efforts have been made to establish more sensitive techniques and to define existing techniques more precisely (see publication #6, and papers in preparation). The lack of adequate, trained immunologists and corresponding equipment, however, has resulted in these efforts being sporadic and less than satisfactory.

b. Characterization of the long-term immune response, particularly to tetanus and diphtheria immunization. Considerable information has been accumulated especially as regards tetanus immunity (see publications #1, 2, 3, 4, 15 and 17, and papers in preparation). Comparable information on diphtheria immunity is meagre, however (see publication #13).

c. Reactions to immunization have been a matter of concern since they must be considered in establishing guidelines for antigen purification, antigen dose, schedules of injection, etc. Some information on reactions and possible ways to avoid them has been accumulated and published (see publication #15).

d. Characterization of the antigen used: Although it may be widely assumed -- on the basis of the studies on tetanus toxoid carried out some 20 years ago -- that tetanus toxoid is quite fully characterized, this is by no means the case (see e.g. Tomita and Feigen 1969). It is axiomatic that no precise immunological studies of any antigen can be interpreted with finality unless the characteristics, homogeneity, etc. of the antigen are fully understood. Also it is axiomatic that unless an antigen is used in the purest possible state the possibility of reactions when it is administered to man is increased. For both the practical and the theoretical reasons given above, efforts have been made in this study to characterize tetanus toxoid physically, chemically, and to some extent physiologically, as far as possible (see publications #7, 9 and 14).

e. Potency testing of typhoid vaccine. This unrelated topic has been included in the activities of the contract for the past three years, because (1) the DOD program for typhoid prophylaxis has become highly dependent upon the ability correctly to evaluate the potency and efficacy of a particular type of typhoid vaccine (acetone-killed and dried vaccine); (2) although no satisfactory method for testing the potency of this vaccine in the laboratory has been established, one particular method (prevalent in the U.S.) has been widely claimed to be consistent with field results and therefore 'acceptable' as a method; (3) data developed first in other laboratories and substantiated by studies in our own laboratory throw considerable doubt on this claim; and (4) the Responsible Investigator has had long experience in this particular field and can therefore undertake a study of this type, particularly in a laboratory that already does this type of testing regularly, without preliminary tooling up. However, since this subject is not pertinent to the basic terms of the contract, it should be reviewed for possible consideration as a separate contract. One unpublished report to the WHO (BS/66.861) has been submitted.

The above review and summary of the purposes of the contract and the approaches taken are presented in order to provide a clearer background to the relatively fragmented information included in any given year's annual report. This year's results follow:

RESUME OF PAST 11 MONTHS' WORK

1. Factors influencing the early primary immune response.

a. Secondary response 1 month vs. 2 months after primary injection. Further analysis of the immune responses observed in the group of 150 humans described in the 1967 and 1968 reports, who received two doses each of one of four toxoids differing in their content of antigen and aluminum phosphate, disclosed no significant difference in the response seen in the groups receiving their second dose at 28 days or 56 days after the first dose.([illegible]). However, the numbers are relatively small, the difference in intervals and the intervals themselves are relatively short, and more data are required in order to determine whether or not the inference that might be drawn from this particular set of data is valid. In any case, it may be contrasted with the striking evidence (see below) of the effect of intervals at less than vs. more than 1 year, between the first and second doses.

b. Antibody level at 1 year vs. 1 month after 2nd inoculation. It was also noted that the rate of fall of the antibody level observed one month vs. 11 months after the second dose of antigen was on the order of 50%. Since many observations suggest that most of the fall-off occurs in the early portion of this period, it may be tentatively assumed that even during the latter part of the first year of a two-dose "primary" immunization, the "half-life" of the residual antibody is probably more than one year. (see Table 1).

Table 1

Geometric Mean Antitoxin Levels
After 1 and 2 Doses of Tetanus
Toxoids AL6A, B, C or D

Toxoid Lot	Content per Dose Antigen	Content per Dose Al+++	* $AlPO_4$	G.M.T. of Antitoxin at 28 days	G.M.T. of Antitoxin at 56 days[1]	G.M.T. of Antitoxin at 56 days[2]	G.M.T. of Antitoxin at 1 year
AL6A	5 Lf	0.2 mg.	0.9	.0038*	.0066	.17	.056
AL6B	25 Lf	0.2 mg.		.0085	.015	.56	.29
AL6C	5 Lf	1.0 mg.	4.52	.0080	.019	.23	.12
AL6D	25 Lf	1.0 mg.		.029	.032	.69	.40

* Antitoxin units/ml of serum

1 Second dose on Day 56

2 Second dose on Day 28

* IND filed in 1966 gave the following conc—

$AlPO_4$	Al+++
0.77	= 0.17
0.77	
3.86	→ 0.8538 mg
3.86	

c. Antitoxin levels 1 month and 1 year after a single injection of tetanus toxoid. The second study group of 40 subjects, inoculated with a single dose of the maximally antigenic toxoid preparation AL6D in October 1967, and given no further tetanus toxoid injections in the ensuing year, was bled in November 1968. Of the subjects originally inoculated, some had disappeared, some had not had every scheduled bleeding in the early part of the study, and two had received tetanus toxoid inoculations in the interim. The final group available and eligible for analysis came to a total of 18 subjects. All 18 carried measurable tetanus antitoxin titers (Table 2), and the geometric mean titer of the group was 0.04 unit/ml, approximately four times the generally accepted "protective threshold". These results are in striking contrast to established conventional thinking about immunization with inactivated antigens -- e.g., that no one was immune to tetanus until he had completed his entire primary series of inoculations with tetanus toxoid. This concept may have been correct in the days when tetanus toxoids were of low potency and the residual titers fell off rapidly, as Gold (1938) showed years ago. It would appear, however, from the above data -- limited though they are -- that a beginning could be made in re-defining the basic concepts of immunization schedules; using suitably designed preparations, it appears possible to accomplish higher and more durable immunity with fewer injections, without having to resort to preparations of unproved safety in man. Thus tetanus immunization might be reduced to a single starting dose, a second dose perhaps a year later, and subsequent doses at long intervals which could be estimated with reasonable accuracy, even though they could only be determined with precision by rather prolonged studies. However, it must be noted that the unexpectedly good results obtained in this study group, started in 1967, require further confirmation (see below).

d. Age and immune response. When the antitoxin levels at 28 days after inoculation of previously non-immune subjects are plotted in relation to the age of the subject, a striking inverse correlation between age and response is seen (see Fig. 1). The same inverse relationship was also apparent in the 1-year bleedings. The numbers are small, but the implication is clear that somewhere in the mid-teens a maximal -- and perhaps a more uniform -- antibody response may be seen, which slowly decreases with increasing age. Thus it becomes impossible to interpret data on the EPIR unless information is obtained on the importance of host factors in determining the responses observed.

e. Bimodal response at 28 days: The appearance of a bimodal distribution of the 28 day antibody titers in both the 1966 and the 1967 study groups in Colombia was noted in last year's report.

Table 2

Antitoxin Titers Following a Single Dose of Tetanus Toxoid AL6D, in Subjects with No Prior Contact with Tetanus Toxoid

Titer AU	No. of Subjects at Indicated Interval Following Toxoid Injection 28 Days	13 Months
<0.0025	-	-
0.0025-.005	1	2
0.005-.01	2	0
.01-.02	5	3
.02-.04	2	2
.04-.08	0	5
.08-.16	2	3
.16-.32	7	2
.32-.64	3	1
.64-1.3	4	-
1.3-2.5	0	-
2.5-5	1	

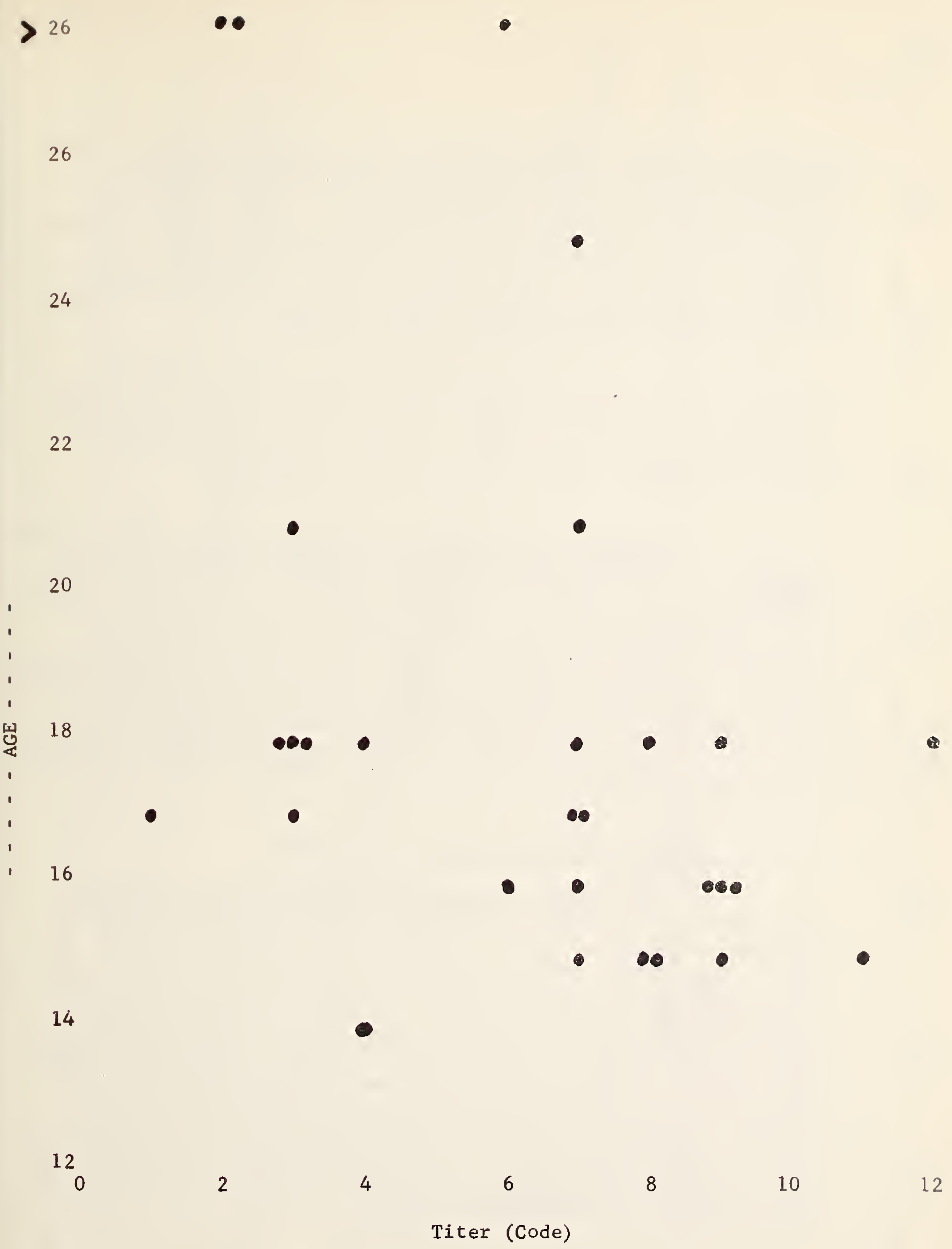

Fig. 1 Age vs. Tetanus Antitoxin Titer at 28 days in previously unimmunized subjects.

It appears to indicate the presence of two populations in each of the groups studied -- "good responders" and "poor responders". The significance of such a bimodal pattern of response would be considerable if confirmed. It might be due to some factor related to the menstrual cycle (all subjects having been females) -- or perhaps to variations in the STH level, with (in this group) the menstrual cycle; Pierpaoli and Sorkin (1969) have shown that STH has a controlling effect on the immune response.

In any case, the bimodal distribution of immune responses appears to be a more and more frequently observed phenomenon (e.g. see White et al 1969) and hence it requires explanation. Specific experiments have been designed to uncover possible influence of genetic differences, age, race and sex, by "piggy-backing" on existing community studies in Louisiana and Colombia.

f. Relation of third dose of toxoid to degree of response obtained: Following the administrative decision a few years ago, approved by the Commission on Immunization, to reduce the third dose of adult type tetanus-diphtheria toxoid in the military program to 0.1 ml instead of 0.5 ml, doubts have been raised as to the evidence for the effectiveness of this lower dose. Therefore a study to determine its effectiveness has been planned, in conjunction with the Preventive Medicine Division, Office of The Surgeon General, and a suitably selected advance training post where individuals scheduled for such a third dose could be found in sufficient numbers for a study. However, no sera have been received at this laboratory to date, for titration.

2. Prevalence and maintenance of immunity.

a. The study in collaboration with Dr. T. C. Peebles, summarized in last year's report, has been published (see below). Re-examination of the data, according to a suggestion from Dr. J. Ipsen, has shown a clearcut relationship (see Fig. 2) between the slope of the antitoxin level/interval since last inoculation curve, in relation to the number of doses of toxoid containing antigen which had been administered to the subjects. It would appear that long term maintenance of immunization might be considerably simplified, after the first few doses of the antigen had initiated the mechanism responsible for the extremely slow fall-off of the residual antibodies in question. The nature of this mechanism has intrigued immunologists for years, and has yet to be explained; when a suitable lead appears, it urgently needs investigation, for both practical and theoretical reasons.

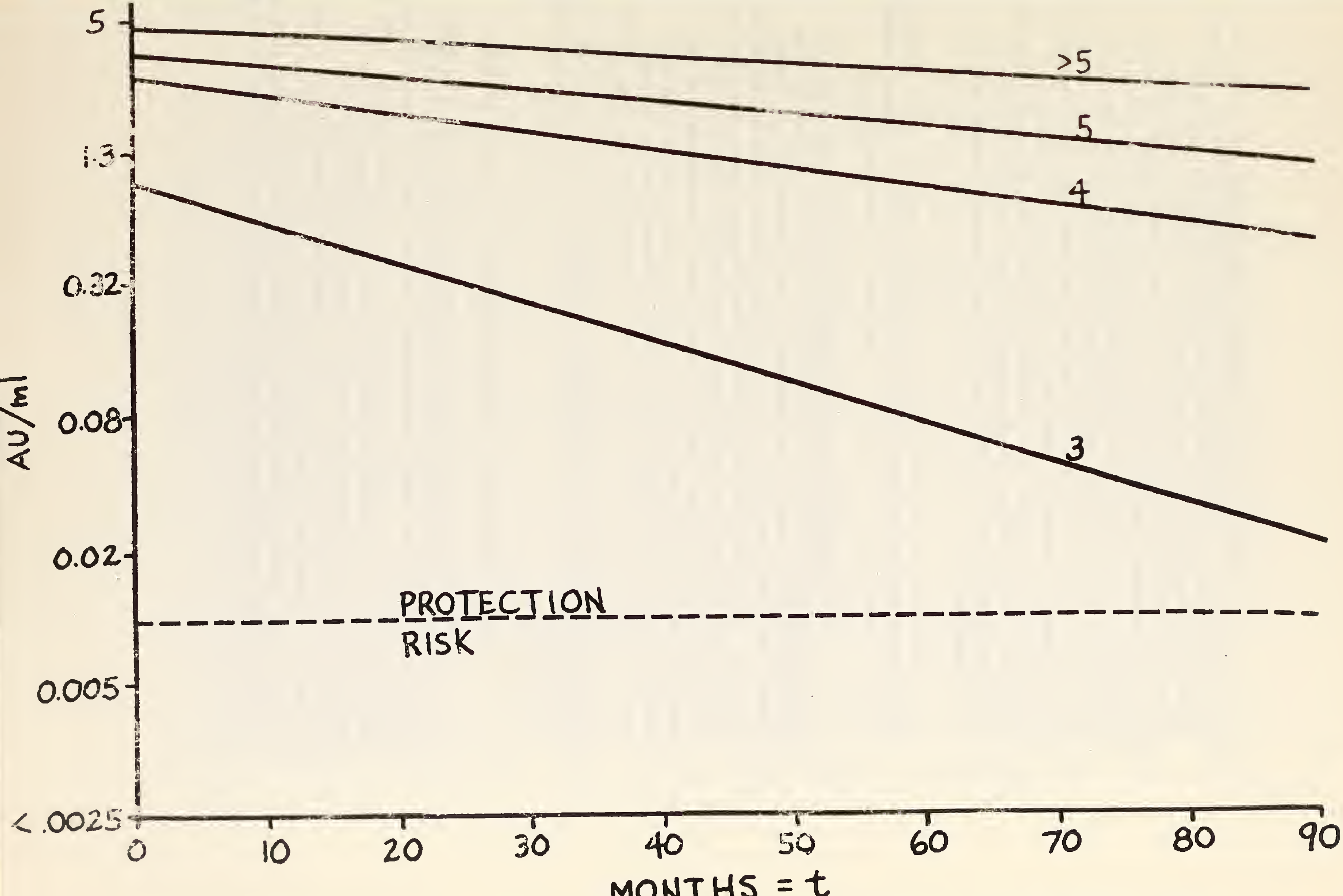

Fig. 2. Fall-off Slope of Tetanus Antitoxin levels after last of 3, 4, 5 or >5 injections of DTP

b. Maintenance of immunity to diphtheria. A study has been initiated in collaboration with Dr. Peebles, parallel to the one already published on tetanus antitoxin levels, to measure diphtheria antitoxin levels in relation to interval since last injection, number of injections, etc. This study would be useful (a) to supplement the very meagre information existing on this subject, and to provide a basis for determining the optimal intervals for routine booster inoculations of diphtheria toxoid; and (b) to test the validity of the assumption that the data described above based on tetanus toxoid immunization can be treated as a generalization, at least with regard to immune responses to protein antigens. Information on the validity of such a generalization would be extremely valuable in making more general plans for long term immunization.

Blood samples are now being collected to support the diphtheria antitoxin study and they will be titrated as soon as a large enough number is accumulated to justify their testing economically.

c. Persisting antitoxin levels after 2 vs. 3 doses of tetanus toxoid. Further analysis of the antitoxin levels in sera obtained at the close of the "Guachené" study (Newell et al 1966) showed that the final mean serum antitoxin level of women who only received two injections spaced more than one year apart was similar to that of the subjects who received three injections at short intervals and approached that of women given three injections at long intervals. If these findings are valid and of general applicability, then two widely spaced injections can give an optimal result. Indeed, the data suggest that an exceptionally long interval between 2 injections may make a third injection essentially superfluous. If such a finding were confirmed, it could introduce a new dimension into immunization programming, and deserves detailed investigation.

3. Purification and characterization of toxins and toxoids:

a. Characterization of the components of tetanus toxin. The major characteristics of the electrophoretically fast moving component of tetanus toxin were summarized last year. Characterization of the smaller, electrophoretically slower component has been more difficult. It appears to contain the predominant portion of the tetanolysin component of tetanus toxin, but this component appears to be quite unstable. Use of ammonium sulfate at any stage in the fractionation scheme will inactivate it. Much activity is lost through Sephadex separation. Concentration on an Amicon diaflo filter results in the loss not only of hemolytic activity but also of considerable flocculating activity.

The hemolytic activity is present in what we first characterized as "Fraction III" (Latham et al 1965). This fraction shows a partial cross-reaction in agar-gel diffusion with the major toxoid component, but little or no immunogenic potency. It is apparently identical with the low M.W. "degraded toxin" described three years ago by Peetoom (1967) and more extensively studied recently by Cohen et al (1969). While the importance of tetanolysin as such has not been demonstrated, the importance of identifying and characterizing this component immunogenically is considerable, since it is in essence a contaminant to the 7S "tetanospasmin" toxin (or its corresponding toxoid), and in immunological titrations it gives misleading flocculation reactions. We have recently found it possible to manipulate toxin production in such a way that the amount of the "Fraction III" is very much greater than usual (see Table 3). This serves to underline the great variation in composition that may occur between tetanus toxins (and their derived toxoids) prepared under different conditions. For this practical reason, aside from the fundamental value of characterizing any component in an antigenic complex used in man, we have continued to attempt to accumulate characterizing data on the two molecules. Whether or not the smaller "Fraction III" tetanolysin-associated molecule is identical with the peripherally acting toxin described by Feigen several years ago, and recently purified by Tomita and Feigen (1969) has yet to be determined. At all events, it is quite apparent, in the light of our work, that of Feigen and associates, that of Cohen and associates, etc., that "tetanus toxin" is not the simple molecule that it appeared to be 20 years ago.

b. Effect of aggregation on antigenicity of toxoids. Through the work of various investigators it is predictable that aggregation of an antigen, such as a toxoid, should markedly enhance its antigenicity. Thus the well known loss of antigenicity of fluid diphtheria toxoid in Great Britain, described by a M.R.C. committee seven years ago, was presumably due to the elimination of aggregated toxoids from the preparation, and the concentration of a highly purified "monomer", soluble, relatively non-antigenic toxoid analogous to the monomer form of bovine serum albumin used by Frei et al (1965) and others in production of tolerance. However, separation of a pure "monomer" diphtheria toxin or toxoid has not proved to be regularly reproducible. An attempt was made to obtain a highly purified diphtheria toxoid with these characteristics by utilizing the procedure described by Goor and Pappenheimer (1967) for diphtheria toxin. However, it was found that under these conditions, the toxoid adhered to the column along with most of the impurities. Obviously the alterations produced in the diphtheria toxin molecule by formaldehyde treatment changed its characteristics. Therefore we have

Table 3

Comparison of the Relative Content of Two Forms of Tetanus Toxin under Different Culture Conditions

	Standard Volume Culture		Small Volume Culture	
Potency	Fraction II (Pool 1100-1675 ml)	Fraction III (Pool 1675-2200 ml)	Fraction II (Pool 1200-1500 ml	Fraction III (Pool 1750-2000 ml)
MLD/ml	4×10^5	0.5×10	2.7×10^6	2.7×10^2
Lf/ml	45	15	150	150
Kf (min) at 50 Lf	10	(150)*	10	145

* Due to the concentration Kf time not relevant

gone back to purification of toxin on DEAE cellulose. Detoxification and aggregation with glutaraldehyde will be undertaken on the appropriate eluted fraction.

The highly antigenic Wellcome Research Laboratories "lysin-treated" diphtheria toxoid shipped to us last year (see 1968 report) was shown to contain considerably more "dimer" than is normally found in our preparations; both components were separated on Sephadex G-200. Each preparation was approximately equal in immunogenic potency, and exceeded the potency of our standard toxoids by about 4- to 8-fold. Thus, on the basis of this one preparation studied, itis evident that antigenicity may reside in some as yet undefined characteristics of the toxoid molecule. (For additional evidence that aggregation alone does not predetermine antigenicity, see Dvorak et al 1969).

4. Techniques for measurement of antibodies and antigenic potency.

a. Study of the nature of false positive tetanus antitoxin titers obtained in passive hemagglutination tests: In reexamination of the data obtained from the study of 150 volunteers first inoculated in 1966 in Cali, Colombia, the ratio of hemagglutination titers to antitoxin titers in the blood samples obtained 21 days as compared to 56 days after a single inoculation of either Toxoid AL6A, B, C or D, was determined. The results are summarized in Table 4. It is clear that the HU/AU ratio is much higher in the early than in the later bleedings, and that this difference is most marked in the subjects who have received the less antigenic toxoid preparations. These findings strongly suggest that the hemagglutinating antibody is -- as might well be predicted -- an early IgM type of molecule. On the other hand, the very widespread prevalence of hemagglutinating antibodies in the absence of detectable antitoxin (see previous annual reports) suggests that they may also represent a relatively non-specific response, perhaps to some as yet unidentified antigen which cross-reacts with tetanus toxin or toxoid. Characterization of the antibody components involved in the HA and AU tests in a number of representative sera is underway, and experimental attempts to reproduce the pattern in rabbits will be undertaken later this fall. The importance of characterizing this phenomenon is because (1) hemagglutination is commonly recommended as a technique for measuring tetanus antitoxin titers (an assumption which can be accepted for clinical purposes only if it is thoroughly validated) and (2) the apparent lack of correlation between the two types of measurement, in certain individuals, raises the possibility that some studies on the serologic prevalence of tetanus immunization may be subject to false interpretations.

Table 4

Hemagglutinating vs. Antitoxic Antibody Titers in Subjects Inoculated with Tetanus Toxoids AL6A, B, C and D 21 vs. 56 Days after a Single Injection

	Toxoid A				Toxoid B				Toxoid C				Toxoid D			
	21 days		56 days		21 days		56 days		21 days		56 days		21 days		56 days	
No. of Subjects	13		13		13		13		13		13		10		10	
	HU	AU	HU	AU	HU	AU	HU	AU	HU	AU	HU	AU	HU	AU	HU	AU
G.M. Titer (Code)	5.6	0.23	9.23	6.38	6.46	0.77	10.46	8.23	5.85	0.85	9.77	6.85	7.90	1.80	10.6	8.90
Ratio, HU/AU	24.4		1.4		8.4		1.3		6.9		1.4		4.4		1.2	
Fold Decrease in Ratio, 21 d. to 56 d.	17.5				7.2				4.5				3.8			

5. Studies on Typhoid Vaccine.

Attempts to reproduce the antigen-suppressive effect reported by Franzl for casein hydrolysate were successful. Further study disclosed that the casein hydrolysate was pyrogenic. In line with the negative role of the supernatant fluid of acetone-killed and dried vaccine shown by Melikova and Lesnjak (1967), our present studies are directed towards determining whether or not the presence of soluble endotoxins in a typhoid vaccine -- whether they are typhoid-specific or not -- may decrease rather than increase the apparent antigenicity of the vaccine, especially if the route of inoculation is intraperitoneal. Preliminary experiments are in process, to define the experimental pattern for testing this assumption.

6. Publications.

A summary of the scientific papers published under the contract since its inception is given below (excluding abstracts which have been superseded by a complete paper):

1. Levine, L. and Wyman, L. Tetanus Immunity in the North and South. J.A.M.A. 187: 518-520, 1964.

2. Gottlieb, S., McLaughlin, F. X., Levine, L., Latham, W. C. and Edsall, G. Long Term Immunity to Tetanus - A Statistical Evaluation and its Implications. Amer. J. Pub. Hlth. 54: 961-971, 1964.

3. Levine, L. and Wyman, L. A nationwide Serum Survey of U. S. Military Recruits, 1962. V. Serologic Immunity to Tetanus. Amer. J. Hyg. 80: 314-319, 1964.

4. Levine, L. and Wyman, L. Survey of Immunity by Serologic Methods: Results of Three Successive Surveys of Samples of the Massachusetts Population for Diphtheria and Tetanus Antitoxin. New Eng. J. Med. 272: 23-26, 1965.

5. Edsall, G., Levine, L., Macnaughton, J. S., Latham, W. C. and Hurley, L. Dose and Time Relations of Precocious Immunity and Antibody Immunity to Tetanus Toxin in Mice. Fed. Proc. 24: March-April 1965 (Abstract).

6. Levine, L. and Wyman, L. The Flocculation Test and the Law of Mass Action. J. Immunol. 94: 586-591, 1965.

7. Latham, W. C., Jenness, C. P., Timperi, R.J.K., Michelsen, C.B.H., Zipilivan, E. M., Edsall, G. and Ley, H. L., Jr. Purification and Characterization of Tetanus Toxoid and Toxin. I. Fractionation of Tetanus Toxoid By Gel Filtration. J. Immunol. 95: 487, 1965.

8. Levine, L., McComb, J. A., Dwyer, R. C. and Latham, W. C. Active-Passive Tetanus Immunization. Choice of Toxoid, Dose of Tetanus Immune Globulin and Timing of Injections. New Eng. J. Med. 274: 186-190, 1966.

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13. ABSTRACT

A single dose of a maximally potent adsorbed tetanus toxoid, prepared in conformity to U. S. Public Health Service Minimum Requirements and causing no undue reactions, induced a measurable antitoxin level in 50% of the subjects within 14 days, and a protective antitoxin level (i.e. >0.01 antitoxin unit/ml) that was still present in 16 of 18 subjects over 1 year later. (U)

The 28-day and 1-year antitoxin levels in these subjects showed a striking inverse relationship to age (range 14 to 39 years). The 4- to 5-year residual antitoxin levels after standard tetanus immunization in another group showed a similar (though less striking) inverse relationship. (U)

Two doses of tetanus toxoid, given more than 90 days apart, yielded a residual antitoxin titer, 4-5 years later, approximately equal to that following 3 doses of toxoid irrespective of the intervals in the latter group. Analysis of residual antitoxin levels in children showed a clear inverse relationship between number of injections and slope of the antitoxin fall-off rate with time. (U)

Characterization of the components of tetanus toxin and toxoid--shown to be complex by recent work of others besides ourselves-- has been facilitated by the finding of a culture technique giving much higher yields of the smaller antigen molecule associated with tetanolysin and probably with Feigen's "peripherally acting" toxin. (U)

The presence of soluble endotoxin in typhoid vaccine was implicated as possibly distorting potency tests of the vaccine. (U)

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ANTIBODY STRUCTURE AND FUNCTION

ANNUAL PROGRESS REPORT

by

Herman N. Eisen, M.D.

August 1969

(For the period 1 July, 1968 to 31 July, 1969)

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FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care", as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

SUMMARY

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Annual Report (1 July 1968 - 31 July 1969) to the Commission on
Immunization of the Armed Forces Epidemiological Board

Among about 500 mouse myeloma tumors screened in the past 2 years (here, at the N.I.H., and at the Salk Institute) for their production of immunoglobulins that bind 2,4-dinitrophenyl (DNP) haptens selectively, two have been found with substantial affinity for these ligands. The two active proteins (nos. 315 and 460) have now been produced here in large quantity by serial transplantation of the coresponding plasmacytomas through several thousand BALB/c mice, and the purified proteins are now being compared in detail with respect to their function and structure. Both are polydisperse in serum, but are readily isolated as purified 7S monomers by methods which are similar to those used for isolation of conventional anti-DNP antibodies. Each of the purified 7S proteins has 2 identical and homogeneous binding sites/molecule of 150-155,000 mol. wt., each site being situated in an Fab fragment of 55,000 mol. wt. Differences between the binding sites of 315 and 460 are readily apparent from their differing affinities for a variety of ligands. Their patterns of cross reactions were basically similar to those of conventional antibodies (e.g., rabbit anti-2,4-DNP and anti-2,4,6-TNP, and guinea pig anti-2,6-DNP). Two unusual cross reactions, with 2-methyl-1,4-naphthaquinone and 5-acetouracilcaproate, were discovered with the myeloma proteins and then found also to be displayed by the conventional antibodies, which supports other evidence that the binding sites of these myeloma proteins are authentic representatives of the sites on conventional antibodies. Proteins 315 and 460 also differ significantly from each other in amino acid composition of both their heavy and light chains and in some of their end groups. Study of the detailed sequences of the V regions of these chains has begun.

INTRODUCTION

While much has been learned in recent years about the structure of antibodies and their function, there is little known -- beyond localization of different functions in Fab and Fc fragments -- of the structural basis for antibody function. For example, there is total ignorance of what residues make contact with specifically bound ligands, of how much variation in amino acid sequence is associated with different binding affinities, etc. Unlike enzymes, where comparable information is often obtainable, the difficulty with antibodies is their obvious heterogeneity. The heterogeneity of populations of antibody molecules is, moreover, known only in a gross qualitative sense, with no knowledge for any particular antibody preparation of the number of homogeneous sets it contains, or their frequency distribution. For this and a variety of other reasons, the past 2-3 years has witnessed an intensive search in several laboratories for means of obtaining homogeneous antibody preparations.

In this laboratory we have carried out this search by screening myeloma proteins (each of which is a homogeneous immunoglobulin produced by a clone of neoplastic plasma cells) for antibody activity. So far we have identified 1 human and 7 mouse myeloma proteins with anti-hapten activity. Two of these proteins, with highest affinity for DNP-ligands, have been subjected to extensive study in the past year. Both proteins are IgA immunoglobulins and are produced by transplantable mouse plasmacytomas.

Protein Production. One protein (315) is secreted by the tumor called MOPC-315, and the second '460) is formed by tumor MOPC-460. Each tumor has been carried separately by transplantation at approximately 3 week intervals into fresh BALB/c mice, the donor animals being exsanguinated when their tumors have grown to massive size (ca. 5 gm in a 25 gm mouse). Several thousand transplants of MOPC-315 have yielded several liters of serum containing about 15 gm protein 315. MOPC-460 has been carried on a smaller scale; about 200-300 ml of serum are now on hand, corresponding to perhaps 1 gm of protein 460.

PROTEIN 315

Purification. By exploiting its specific reactivity with the DNP group, this protein has been isolated in purified 7S form by methods analogous to those used in purification of conventional anti-DNP antibodies; it is first precipitated from serum with DNP- or TNP-HSA; then the washed precipitate is dissolved in 0.1 M DNP-glycine, reduced with .005 M dithiothreitol, alkylated with a 10% molar excess of iodoacetate, and by combined ion exchange steps, involving a DEAE-cellulose plus Dowex-1 (x8) column, protein 315 is finally collected free of DNP-HSA and DNP-glycine. Yield is generally about 50% of the protein in serum, which is usually about 7-9 mg/ml, based on quantitative precipitin analysis.

Purity. No contaminants are evident when 315 is examined by gel diffusion or immunoelectrophoresis with rabbit-anti whole mouse serum as reagent. Yields of light and heavy chains, and of Fab fraction, together with corresponding molecular weights (see below), are all consistent with >95% purity.

Physical Properties. Yphantis high speed equilibrium centrifugation has been used to establish the following molecular weights:

7S monomer (2L + 2H)	155,000
Heavy Chains	55,000
Light Chains	21,000
Fab Fragment	55,000

The Fab fragment, obtained by tryptic digestion, was recovered in 65-70% yield; hence, there are 2 Fab fragments per 7S monomer (2 x $55{,}000 \cong 155{,}000/.65$).

Combining sites. Equilibrium dialysis established 1.5-1.9 binding sites per 155,000; the lower value was found in experiments performed at low protein concentrations (~40 μg/ml), and the higher at high protein concentration (0.5 mg/ml). Such a concentration dependence has not previously been observed in measurements of antibody binding sites with IgG antibodies; it is probably due here to the non-covalent binding of light to heavy chains in this IgA protein. H. Grey, for example, has just found with protein 315, as he did previously with 6 other mouse IgA's, that light chain monomers and dimers dissociate from the whole molecule ($L_2 H_2 \rightleftharpoons L + LH_2$ or $L_2 H_2 \rightleftharpoons L_2 + H_2$) in acid urea starch gel electrophoresis. If they also dissociated to some extent in neutral, dilute salt solution, the 7S monomer could exist during equilibrium dialysis as a mixture of univalent and bi-valent molecules, and the average valence would depend on extent of light chain dissociation, which would depend in turn on total protein concentration. An alternative possibility is that at low, but not at high concentrations, the protein undergoes reversible denaturation.

Corresponding to the 7S monomer, the Fab fragment split from it by trypsin digestion, had a valence of 0.8 (not 1.0), when measured at low concentration; again, this could represent the existence of a mixture of a univalent fragment, and a dissociated one that lacks a binding site ($L.Fd \rightleftharpoons L + Fd$).

The yield of Fab (about 65-70% of the 7S monomer) and its number of sites per fragment, as well as the valence value (1.9) found for 7S at highest protein concentration tested, show, finally, that this IgA myeloma is basically symmetrical with 2 potential combining sites per molecule (1 per Fab), as is true for all other 7S antibodies so far examined. Further evidence in support of this view is given below from recent results with protein 460, and the mouse IgA protein.

Specificity and Affinity. As we reported earlier, the intrinsic affinity of 315 for ε-DNP-L-lysine is high (2×10^7 M^{-1} at 4°), and the same affinity value is found under conditions where the extrapolated value for valence varies from 1.5 to 1.9.

The binding of a large number of different ligands to 315 has been tested by competitive equilibrium dialysis, in which the reference ligand was ^{3}H-ε-DNP-L-lysine and the competitors were unlabeled compounds. The object here was to look for some naturally occurring substance that might be bound better than DNP-lysine; this would then fit with the plausible view that the high incidence of anti-DNP activity among myeloma proteins, especially among mouse IgA, is due to spontaneous immunization by a natural immunogen and then random neoplastic change among the antigenically stimulated cells when mineral oil is injected to raise plasmacytomas. The specific binding of DNP in this view would represent a cross-reaction.

Among approximately 50 substances tested (nucleic acid bases, nucleosides, coenzymes, synthetic purines, etc.), the only respectable binding was exhibited by 2-methyl-1,4-naphthaquinone (menadione or Vit. K_5), whose intrinsic binding constant was 6×10^5 M^{-1}, or about 1/15th that of ε-DNP-L-lysine. The relatively strong binding of this naphthaquinone suggests that a vitamin K-like molecule in bacterial flora could, perhaps, represent the natural immunogenic material, whose antibodies cross react with nitrophenyls. Further work along this line is being pursued.

The binding of menadione was startling at first, as its structure appears so different from that of 2,4- and 2,4-6 nitrophenyl ligands, that otherwise are bound specifically by protein 315. A number of conventional antibodies were therefore examined, and rabbit (IgA) anti-2,4-DNP anti-2,4,6-TNP, and guinea pig anti-2,6-DNP were all found also to bind menadione quite strongly. But in all cases, the binding was less good than that of their respective homologous nitrophenyl ligands.

Amino Acid Sequences. The 315 L chain seems to have an asp or glu N-terminus and leu is the C-terminus. Because a second amino acid is not obtained with carboxypeptidase A, the penultimate residue (next to leu) is very likely carboxymethylcysteine (CM cys), produced by reduction and alkylation during preparation of the separated chains. There are 2 met residues per chain and efforts are still underway to find the best solvent conditions for separating the 3 fragments formed by CNBr digestion.

The heavy chain has a tyrosine C-terminus and pyrrolidone carboxylic acid (PCA) appears to be the N-terminal residue, as in most other heavy chains. CNBr digests of heavy chains have also been prepared and most effective solvents for separating the anticipated 6 fragments (5 met residues per chain) on Sephadex columns are being studied.

PROTEIN 460

This IgA protein has been produced in much smaller quantity than 315, but the characterization has proceeded smoothly. Prepared exactly in the same way as 315, its 7S monomer has essentially the same molecular weight, viz., 150,000. Its heavy chain has MW 50,000, and light chain is 23,000. Entirely consistent with these values is the weight ratio of fully reduced and alkylated light and heavy chain separated by gel filtration in dissociating solvents. As with 315 and other 7S molecules, protein 460 also has 2 light and 2 heavy chains per 7S molecule.

By means of equilibrium dialysis protein 460 was found to have moderate affinity for ε-DNP-L-lysine ($K = 3 \times 10^5$ M^{-1}), and its binding sites are homogeneous. Unlike 315, however, its valence is clearly 2.0/150,000 molecular weight. Because affinity was relatively low, equilibrium dialysis had to be carried out at high protein concentration (about 0.5 mg/ml), and this may account for the clear finding of 2 sites per molecule. However, this molecule is probably much like 315, and perhaps it also would exhibit a smaller number of sites per 7S if it could be tested at lower concentration as with 315. This possibility cannot be realized, however, with DNP-lysine as ligand (because affinity it not sufficiently high); it may be possible through the use of 2,4-dinitronaphthol, which has turned out to bind to 460 about 10-fold better than DNP-lysine ($K = 3 x 10^{6} M^{-1}$).

Spectral changes of bound ligands has also been examined with protein 460, and 2,4-dinitronaphthol undergoes a particular striking quantitative change, but quantitatively the shift is to the same longer wavelength (max at 470 mμ) observed in the specific binding of all nitrophenyls to antibodies.

Because 460 binds 7-sulfonate-2,4-dinitronaphthol as well as the non-sulfonated analogue we have prepared the following derivative for attempts at affinity labeling of the active site:

If effective with 460, it would be of considerable interest to use this reagent with 315, as the same affinity label in both proteins could afford a particularly incisive way to compare segments of their chains in the vicinity of their respective active sites.

The composition of 460 and 315 and their respective chains are markedly different (see Table 1). This is not at all unexpected, because (as shown earlier in this laboratory) conventional anti-DNP antibodies of different affinity can also differ considerably, even when isolated from an individual animal.

CHAIN COMPOSITION OF MOUSE IgA MYELOMA PROTEINS WITH ANTI-DNP ACTIVITY ‡

	Light Chains		Heavy Chains	
Residue	315	460	315	460
Lys	10	13	25	38*
His	5	5	8	7
Arg	5	8	13	15
CM Cys	5	5	16	18
Asp	17	23	49	41
Thr	24	19	48	45
Ser	17	27	53	49
Glu	17	19	53	51
Pro	12	11	43	45
Gly	17	14	48	40
Ala	13	8	23	25
Val	14	14	38	37
Met	2	2	5	5
Ile	6	7	16	18
Leu	15	18	54	48
Tyr	3	7	19	23
Phe	9	10	17	12
Trp	-	-	-	-

* Questionable Value.

- not yet determined.

‡ With both proteins there is excellent agreement between amino acid analyses, normalized to Yphanti's mol. wt. of the 7S monomer, and 2x the sum of the light and heavy chains, normalized to their respective molecular weights. Thus these amino acid analyses also demonstrate that the 7S monomer is made up of 2 light plus 2 heavy chains.

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13. ABSTRACT

Among about 500 mouse myeloma tumors screened in the past 2 years (here, at the N.I.H., and at the Salk Institute) for their production of immunoglobulins that bind 2,4-dinitrophenyl (DNP) haptens selectively, two have been found with substantial affinity for these ligands. The two active proteins (nos. 315 and 460) have now been produced here in large quantity by serial transplantation of the corresponding plasmacytomas through several thousand BALB/c mice, and the purified proteins are now being compared in detail with respect to their function and structure. Both are polydisperse in serum, but are readily isolated as purified 7S monomers by methods which are similar to those used for isolation of conventional anti-DNP antibodies. Each of the purified 7S proteins has 2 identical and homogeneous binding sites/molecule of 150-155,000 mol. wt., each site being situated in an Fab fragment of 55,000 mol. wt. Differences between the binding sites of 315 and 460 are readily apparent from their differing affinities for a variety of ligands. Their patterns of cross reactions were basically similar to those of conventional antibodies (e.g., rabbit anti-2,4-DNP and anti-2,4,6-TNP, and guinea pig anti-2,6-DNP). Two unusual cross reactions, with 2-methyl-1,4-naphthaquinone and 5-acetouracilcaproate, were discovered with the myeloma proteins and were found also to be displayed by the conventional antibodies, which supports other evidence that the binding sites of these myeloma proteins are authentic representatives of the sites on conventional antibodies. Proteins 315 and 460 also differ significantly from each other in amino acid composition of both their heavy and light chains and in their end groups. Study of the detailed sequences of the V regions of these chains has begun. (Unclassified).

AD______________________

STUDIES ON THE IMMUNIZATION OF MAN AGAINST PLAGUE (U)

ANNUAL PROGRESS REPORT NO. 19

by

K. F. Meyer, M.D.

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(3) K. F. Meyer, M.D., Director Emeritus, Principal Investigator

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Annual Report No. 19 to the Commission on Immunization of the Armed Forces Epidemiological Board

SUMMARY

A pilot experiment involving 15 langurs vaccinated with 420,000,000 or 42,000,000 freeze-dried viable *P. pestis* E.V. (WR-S) demonstrated that absolute protection against severe, subcutaneous, virulent infection persisted in individual animals for at least 16 months: 10/15 (66%) survived an infection fatal to 3 controls on the 3rd to 6th day. Four delayed fatal infections occurred on the 10th and 12th days and were either complicated by metastatic pneumonia or by pregnancy (1). Resistance is partially reflected by the protective antibody levels: Langurs proven only partially immune had MPI of 10 or above and relatively low HA titers. Despite excellent antibody levels, pregnancy and nonspecific gastroenteritis impaired the immunity. Experiments designed to measure the duration of protection conferred by the U.S.P. killed and the live E.V. 76 (Paris) vaccines are contemplated.

In the search for an E.V. 76 transplant with greater invasive potential, persistence, and correspondingly enhanced protective efficacy, the culture originally received from Madagascar in 1951 was passed through guinea pigs pretreated with iron sulfate. This passage strain, designated E.V. 51f, proved nonpathogenic for guinea pigs by the subcutaneous route in dosages of up to 5 billion, and conferred complete protection with less than 1000 viable organisms. It is lethal, however, to intravenously inoculated white laboratory mice. E.V. 51f possesses all the biochemical, nutritional and antigenic markers which characterize living attenuated *P. pestis* isolates. In Annual Report #18 (1967-68), concern was expressed regarding the results of two vaccination experiments on subhuman primates, since unexpectedly a live plague vaccine accepted by customary standards as "safe" caused vaccination fatalities in African green vervet monkeys with inocula of as low as 2180 viable organisms. In cooperation with the South

African Institute for Medical Research, a third experimental series using 50 Cercopithecus aethiops pygerthrus fully confirmed the results of previous trials. Vaccination fatalities are listed as follows:

(a) Cercopithecus aethiops Ethiopia: D/S - 3/16 or 18%; 160 million, 1 death; 16 million, 1 death; 1.6 million, 1 death.

(b) Cercopithecus aethiops Kenya: D/S - 10/20 or 50%; highest mortality (75%) with 218 million; no deaths with 218 viable E.V. 51f.

(c) Cercopithecus aethiops pygerthrus (South Africa): D/S - 13/50 or 26%; highest percentage (40%) with 100 million and 100,000 viable organisms.

Autopsy lesions and bacteriologic findings resembled those of bubonic-septicemic plague complicated by granuloma formation in the lymph nodes, without hemorrhage, and edema and necrosis of the liver. Animals surviving vaccination exhibited local indurations, lymphadenopathy, and systemic reactions (fever, positive blood cultures, vigorous and significant antibody response). With the exception of the South African species, they were completely protected against severe subcutaneous infection. The antibody response was excellent, as evidenced by MPI levels on the 28th day after vaccination; but in 3 animals, immunity was apparently lost by the 5th month. The reasons for these failures in this species of Cercopithecus will remain unexplained until serologic examinations have been completed.

In langurs (Presbytis entellus), subcutaneous inoculations of the vaccine isolate E.V. 51f in a dosage of 130 million viable organisms caused severe, painful local inflammatory reactions which persisted for more than 2 weeks. Fever and positive blood cultures preceded the deaths of 2/4 vaccinated animals. The antibody response was outstanding in the survivors, which were completely protected against infection. Local reactions due to the vaccine were definitive in 2 young female Cercopitheci included in the test series but were less marked than in langurs. One vervet, clinically ill with a low-grade bacteremia, yielded nonpigmented E.V. 51f in the blood culture for at least 6 days. As in the langurs, low MPI and the early appearance of antibodies correlated exactly with the absolute protection proven by inoculation.

In a single pilot experiment using the bifurcated needle with the intradermal one-puncture vaccination technique on macaques and langurs, the E.V. 51f isolate at first appeared to be safe. It produced no local or systemic reactions in Macaca mulatta. But an opposite interpretation resulted from analysis of clinical tests with langurs. Although local reactions produced by single-puncture vaccination were slight, it was disconcerting that in this highly susceptible species, the vaccine strain proved highly invasive, causing slowly developing generalized infections, reflected by positive blood cultures beginning on the 5th day and rapid enlargement of the regional lymph nodes. The blood-stream was constantly seeded with members of the E.V. 51f population possessing "residual virulence", with consequent secondary pneumonia in the lungs of langurs dying on the 12th to 17th days after vaccination with an estimated dosage of approximately 8000 viable organisms. It was concluded that an E.V. 76 vaccine isolate obtained by guinea pig passage and proven lethal to langurs and Cercopitheci in dosages of 2000 to 8000 viable P. pestis should not be used in clinical trials on human subjects, regardless of the fact that it was harmless to guinea pigs and macaques and conferred solid protection upon langurs surviving single-puncture intradermal vaccination, with only slight local reactions.

In the search for less expensive but equally susceptible mammals other than subhuman primates, the multimammate mouse (Mastomys or Praomys natalensis) was recalled to have been employed advantageously to assay plague vaccines intended for prophylactic human use. In two experiments with 100 rodents, Mr. A. F. Hallett of the South African Institute for Medical Research demonstrated that dosage definitely influences the outcome of vaccination. With doses of 1 million or more organisms, fatalities may be anticipated. Regardless of the vaccination method (subcutaneous or intradermal puncture), doses of 500,000 to 1.7 million organisms conferred 60% relative immunity. Similar studies conducted by Dr. Y. Karimi at the Institut Pasteur de l'Iran, Teheran, using the supposedly highly susceptible gerbil (Meriones tristrami), proved inconclusive. The wild gerbils tolerated subcutaneous inoculations of 507 to 507 million viable E.V. 51f with no local or systemic reactions; HA antibodies developed in the sera of those inoculated with the largest doses, as was the case in guinea pigs. But why these wild rodents proved incapable of developing protection when inoculated with a vaccine proven highly efficacious in other animals remains an intriguing mystery.

A killed plague vaccine was readily prepared from the rehydrated P. pestis E.V. 51f by treating the suspension with formalin and diluting it to a content of 2 billion or 2 million organisms/ml. Guinea pigs inoculated with single doses of the aqueous vaccine afforded no protection. In a group basically inoculated with 2 billion and given single booster doses on the 30th day, 10-20% were protected. Single doses of vaccine in adjuvant #65 protected 80% of the guinea pigs against infection with 870,000 virulent P. pestis 2 months after inoculation. Only 10% protection was attained with 2 million killed vaccine in adjuvant. The protective properties of the killed E.V. 51f vaccine are almost identical to those reported for the U.S.P. vaccine. The chemical killing process deprived attenuated P. pestis E.V. 51f of its invasiveness and multiplication capacity, exerting an influence on the immunity mechanism, but did not alter its antigen structure.

In the course of vaccination experiments using subhuman primates, the disconcerting experiences with E.V. isolates obtained from various sources or by guinea pig passage indicated the necessity of similar tests with a transplant of the original E.V. 76 culture maintained at the Institut Pasteur in Paris. Protective efficacy on guinea pigs is still of a high order. The number of viable organisms capable of protecting guinea pigs is expressed by a PD_{50} (50% protective dose) of 40 using the intracutaneous route and a PD_{50} of 33 by the subcutaneous route. Even in the dosage of 100,000 organisms, the E.V. 76 (Paris) is nonpathogenic for mice inoculated intravenously. The immunogenic and "granuloma-forming" properties in guinea pigs, as well as all the biochemical nutritional marker determinants -- F_1+, pigment negative, CA dependence, uracil independence, Pesticin I negative, Pesticin II+, and fibrinolysis -- are readily demonstrable. Vaccination experiments involving langurs and African vervets, each intradermally inoculated with 26,800,000 viable organisms in 0.1 ml of E.V. 76 (Paris), established that the vaccine induces the development of indurated nodules persisting for 5 days and progressive lymphadenopathy (a node 5 x 3 cm in diameter on the 16th day in one langur). Local signs were accompanied by systemic reactions -- anorexia, 1-2° rises in body temperature, moderate leucocytosis, and diarrhea. Quantitative blood cultures yielded 2 to 16 colonies in 2 young langurs on the 2nd, 4th, and 8th days after vaccination. On the 5th day, lymph node biopsy specimens revealed moderate to abundant growth of the vaccine strain. Significantly high HA titers and complement-fixing antibodies in titers up to 1:128 conclusively contradicted reports that live attenuated plague vaccines fail to induce peripheral antibodies. On the 42nd day after vaccination, immunity tests by subcutaneous infection with 343,000 virulent *P. pestis* revealed an absolute protection illustrated by the absence of local reactions in the skin and regional lymph nodes. The nonvaccinated controls, which died on the 4th day, as well as other langurs used in an experiment to evaluate the immunogenic efficacy of Fraction 1, displayed extensive hemorrhagic, necrotizing dermatitis and lymphadenitis. In Kenya African green vervet monkeys and *Macaca mulatta* local skin reactions developed; these were less marked, however, than in the langurs. Lymph node enlargement was also moderate in comparison to that seen in langurs. Evolution to prominent nodes on the 5th to 16th days, receding in size by the 27th day, differed only in intensity from such reactions in the langurs. Three *Cercopitheci*, bled on the day they revealed body temperatures above 40°, yielded a few colonies of E.V. 76 (Paris) on blood plates or upon enrichment in broth cultures on the 2nd and 5th days. In the macaques, local and systemic reactions were negligible and serologic response was less vigorous than in langurs. The outcome of the immunity tests to be performed 3 months after basic vaccination is awaited with particular interest.

The invasiveness and "residual virulence" of the E.V. 76 (Paris), combined with a high immunity rate but accompanied by definite local and systemic side effects (probably essential for an efficacious live plague vaccine) in langurs and vervets, gives rise to the pertinent question: Is it really "harmless" to man? Clinical trials with carefully supervised human subjects, using the intradermal single- or double-puncture vaccination technique with the bifurcated needle, will provide the answer to the important inquiry: Is the E.V. 76 (Paris) a safe protective live plague vaccine? The fact that the transplant, upon passage through guinea pigs, (like E.V. 51) acquired pathogenicity for mice merits consideration.

The Fraction 1-deficient, nonpigmented living *P. pestis M23* (Burrows), pathogenic for mice upon intravenous inoculation in a dosage of 40 million organisms, is *lethal* to 4/4 intradermally inoculated langurs, It is likewise invasive and pathogenic for vervets but not rhesus monkeys in a dosage of 38 million, which is well tolerated by guinea pigs. Even in smaller doses (1300 to 2200 organisms), this attenuated *P. pestis* isolate has proven highly protective. The conjecture by Burrows (1957): "Does M23 behave in man as it does in the mouse or in the guinea pig?" has been indirectly answered by the high mortality rates in langurs and vervets. Until the pathophysiologic injuries produced by M23 can be better understood, further experiments with an ultimate goal of using this isolate to protect man against plague have been discontinued, and the M23 is classified among the "harmful" living attenuated *P. pestis* isolates.

The conviction that Fraction 1 is the primary protective antigen against infection provoked by wild strains of *P. pestis* has been weakened by the results of experiments with langurs. Four out of six langurs inoculated over a period of 5 months with total doses of 5 mg Fraction 1 in aqueous or adjuvant preparations responded to infection by dying belatedly on the 12th (3) and 14th days. Experiments with Fraction 1 involving vervet monkeys are currently in progress.

FOREWORD

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care", as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences, National Research Council.

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I. Duration of immunity in langurs (Presbytis entellus) vaccinated with living (42 million or 420 million organisms) freeze-dried E.V. 76 (WR-S).

In Annual Progress Report No. 18 (p. 46, Table 8), an experiment was described which involved testing the local reactions and antibody response of langurs to the Saigon transplant of E.V. 76 (WR-S), which had proven inadequately immunogenic in two clinical trials on human subjects. Antibody responses, expressed in HA and mouse protection indices (MPI), in 15 langurs remained high for at least 2 months, but declined by the 3rd month. Since experience had demonstrated that 6 of 7 langurs with such antibody levels could survive severe challenge infections given on the 100th day after vaccination, it was decided to reserve these animals for an experiment to determine the duration of immunity. Over a period of 13 months, antibodies were measured on the 93rd, 126th, 177th (approx. 6 months), and 406 days (approx. 13 months) after vaccination. The average MPI rose from 2.5 on the 28th day to 8.4 on the 406th day; in some individual langurs it returned to normal levels. Hemagglutinins persisted but likewise declined from an average of 1:4554 to 1:1335 in the 13th month. In groups of 8 langurs each receiving a booster inoculation on the 406th day, the elevation of antibodies within 11 days was striking (see Table 1), but by the 85th day after re-inoculation, a decline in titer was again recorded. The antibody recall response to approximately the same dose of vaccine (45 million organisms) as the original vaccination (42 million organisms) was for unexplained reasons more pronounced than in the group vaccinated with the larger dose of vaccine (420 million organisms). Vaccinated langurs' resistance to severe subcutaneous challenge infection (307,000 P. pestis 195/P) given during the 16th month after original vaccination was surprising: 10/15 (66%) survived the infection dose fatal to 3 controls on the 3rd to 6th day. Seven of the survivors exhibited solid immunity, but this protection was relative, as indicated by local skin reaction (SE 231) and draining axillary buboes (SE 249) persisting for 28 days. The fatal infections occurred between the 10th and 12th days and were either complicated by metastatic pneumonia or the other by pregnancy, dangers so frequently observed in human beings in the course of every plague epidemic, ancient or modern. The groups of langurs available for the experiment were too small to gauge the efficacy of the booster inoculations adequately. Only Group I, with a 3/4 versus 1/3 survival rate, exhibited an enhancing effect with the vaccine, which was over 2 years old and perhaps had partially deteriorated. It was more pronounced in Group II, since 3/4 langurs without local lesions and probably all without pregnancy would have survived. The vaccinated langurs, however, possessed some degree of protection; their deaths (due to bubonic-septicemic plague) were complicated by metastatic pneumonia consolidations of varying extent and intensity. No systematic temperature measurements were taken, but from previous experiments it is known that chemotherapy instituted when fever was recorded would have cured the slowly progressing infections which are usually rapidly fatal to nonvaccinated animals.

TABLE 1

Duration of Immunity Observed in Langurs (Presbytis entellus) Vaccinated with Living (42 million or 420 million) Freeze-Dried E.V. 76 (WR-S) With and Without Single Booster Doses During 491 Days (16 months)

	Antibody Titers - Day						Booster		Post-Booster, 45.5 million						Response to Challenge	
Animal	28th			126th			406th		431st			491st			307,000 *P. pestis* sbc	
Number	HA	CF	MPI	HA	CF	MPI	HA	MPI	HA	CF	MPI	HA	CF	MPI	Day of Death, Lesions	S/T
I.																
SE 203	4096	0	4	64	0	16	256	14	8192	0	2	2048	0	7	Survived)	3/4
SE 223	1024	0	2	128	0	12	16	12	512	2	4	2048	0	13	Survived)	
SE 214	4096	2	4	256	0	4	16	12	256	0	5	512	0	10	11. Metastatic pneum)	
SE 249	4096	4	2	128	0	10	4096	7	2048	2	3	4096	0	4	Survived; local)	
SE 274	512	4	1	512	0	10	512	11	* 128	0	12	256	0	15	10. Metastatic pneum)	1/3
SE 276	1024	0	4	256	0	6	128	12	* 32	0	15	(64)	0	15	Survived)	
SE 277	32,768	32	∞	4096	0	∞	4096	5	*4096	0	6	512	0	7	11. Metastatic pneum)	
II.																
SE 261	512	64	∞	256	2	3	256	6	8192	2	∞	256	0	3	Survived)	3/4
SE 239	16,384	4	2	256	2	7	256	6	4096	16	∞	2048	4	1	Survived)	
SE 263	256	2	1	2048	2	2	8192	2	8192	4	1	8192	2	2	12. Pregnant)	
SE 207	64	4	13	32	0	15	32	12	2048	4	∞	256	2	2	Survived)	
SE 231	1024	0	1	256	0	6	128	5	* 128	0	9	128	0	∞	Survived; local)	3/4
SE 245	1024	16	1	1024	0	5	1024	4	*4096	0	5	512	0	5	Survived)	
SE 247	256	2	1	4096	0	3	512	9	* 64	0	4	512	0	9	Survived)	
SE 243	1024	4	1	2048	0	4	512	9	* 64	0	8	256	0	10	10. Metastatic pneum)	

I - 420 million dose
II - 42 million dose
* - not boostered

Before an attempt to correlate serum antibodies and resistance, it should be recalled that the E.V. (WR-S) vaccine invariably stimulated antibodies in rodents and subhuman primates; a dose of 2 billion with 420,000,000 viable organisms reduced the MPI to below 10, or raised the HA to 1:512 in a few human subjects, but a small booster dose of 27.6 million with 8.45 million viable organisms proved ineffective in altering the MPI. U.S.P. plague vaccine and Fraction 1 were effective as booster inocula. By contrast, antibody response in langurs was vigorous with respect to HA and MPI; by the 28th day after vaccination significant HA levels persisted and MPI were below 10. The correlation between MPI and resistance to infection was less striking than in previous experiments when the animals were challenged within 2-3 months after vaccination. Of the 10 survivors, 8 had MPI below 10, in fact 6 had MPI below 5. The past history of langur SE 273 indicates a good response to the vaccination schedule, with a low MPI. For 177 days the MPI of SE 276 was below 10 (at times even below 5), but by the 406th day it had risen to 12 and remained on this level; however, the animal proved resistant despite the high index. Three (SE 249, 274, 243) of five langurs proven only partially immune had MPI of 10 and above and relatively low HA titers. Despite excellent antibody levels following vaccination, pregnancy (SE 263) and nonspecific gastroenteritis (SE 277) impaired the immunity, as indirectly reflected by the serologic titers measured a few days before infection.

Through the courtesy of Dr. Michael J. Surgalla, Chief, Immunology II Branch, Fort Detrick, 4 langurs (weighing 6-8 kg), each inoculated subcutaneously with 45.55 million viable _P. pestis_ E.V. 76 (WR Saigon) and boostered 18 weeks after primary inoculation with 60.5 million viable organisms of the same vaccine, were exposed to aerosol infection (KIM-10, inhaled dose 8.1×10^5). Two non-immunized langurs and 2 rhesus monkeys died on the 3rd day after basic inoculation with lesions of pneumonic plague. Of the immunized langurs, 1 survived but revealed evidence of recovery when the organs were examined following sacrifice 21 days after exposure. Two died on the 10th day and 2 on the 6th. The infective dose was much higher than intended. Nevertheless, some protection was afforded the vaccinated animals. All had MPI which were either complete or 1.

Studies with the attenuated E.V. (WR-S) transplant were discontinued, but the following comment appears to be justified:

> The pilot experiment on langurs confirms the published findings in experiments with guinea pigs. Girard (1948) found that the E.V. vaccine in these animals produced an immunity which lasted for about a year or even longer, though in earlier experiments the immunity disappeared by the 3rd month. According to Korobkova (1956), resistance to infection induced by a single inoculation of live vaccine was preserved for at least 8 months. Otten (1936) observed that the immunity produced in guinea pigs by administration of the Tjiwidej strain was still fairly

satisfactory (?) after six months but markedly decreased after the 9th month. In the experiment described, 4/7 langurs proved resistant when challenged 16 months after single doses of 42 million or 420 million E.V. (WR-S). Previous studies (Annual Report #18, p. 42) had demonstrated that the same vaccine in a dosage of 53,000,000 organisms on the 100th day after inoculation protected 6/7 langurs against fatal but not local plague reactions. Thus absolute protection conferred by live vaccines upon subhuman primates may persist in individual animals for at least 16 months. The resistance is partially reflected by protective antibody levels. By the end of the 3rd month after basic vaccination, the MPI of individual animals began to fluctuate and by the 12th month might reach relatively low levels. Revaccination restored the original level. However, experiments conducted on small groups of langurs failed to furnish conclusive information concerning the booster effect conferred by revaccination, invariably demonstrated with killed vaccines. Furthermore, it must be emphasized that the study on the duration of immunity in langurs was conducted with a vaccine which proved incompletely immunogenic. Hence the experiments must be repeated with E.V. 76 (Paris), preferably using groups of not less than 10 animals inoculated with PD50 dosages for langurs previously determined by titrations. While housing facilities and arrangements for animal care are being developed for such studies, experiments to assess the duration of immunity conferred by this relatively innocuous live vaccine upon guinea pigs have been initiated.

In the course of independent studies conducted by Dr. L. Leshkovich of the Plague Control Institute in Alma Ata (personal communication), a formalinized killed virulent *P. pestis* vaccine administered in two steps protected 50% of the guinea pigs against fatal infection when they were challenged 11 months after basic inoculation. Deaths were delayed for 20 days, in comparison to controls that succumbed to infection between the 4th to 7th days. Autopsy of the survivors disclosed lesions of infection in the form of necroses at the sites of infection, regional lymph nodes and liver, indicative of declining immunity. In the same communication, it is noted that 50% of a series of guinea pigs vaccinated intracutaneously with the E.V. Stavropol vaccine (see Annual Report #17, Table 9, p. 22) died on the 12th day after challenge infection and apparently had lost their protection by the 11th month. Experiments with guinea pigs using alum-coated killed plague vaccines (Annual Report #13, Table IV) proved that immunity, which was absolute one month after basic immunization (2 doses), declined by the 3rd month. Because of these observations, it was recommended that revaccination be performed at 3-month intervals when exposure to infection in endemic areas may be anticipated. More recent observations, if confirmed by additional studies, may justify an extension of revaccination to 6-month intervals or even longer.

II. Studies on pathogenicity and immunogenic efficacy of living E.V. 51f vaccine.

(1) Vaccination of green vervet monkeys (Cercopithecus aethiops subsp.); high vaccination mortality. In Annual Report #18 (p. 50-66), the origin of the live plague vaccine E.V. 51f and the pathogenic reactions observed in African green vervet monkeys (Cercopithecus aethiops) from Ethiopia and Kenya were discussed. At the time the report was submitted, however, the resistance of the 10 Kenya species of Cercopitheci which survived vaccination with 218 to 218 million E.V. 51f had not been tested. The completed experiment is now detailed in Table 2.

As anticipated from the antibody levels (MPI of 5 and below) obtained with the serum of the surviving vaccinated Cercopitheci, protection from subcutaneous challenge infection with 106,000 P. pestis 195/P on the 52nd day was complete. Even the animals vaccinated with the small dose of 218 viable E.V. 51f withstood the infection effectively. None developed lymphadenopathy or local lesions at the site of infection. By contrast, the two controls succumbed to bubonic-septicemic plague on the 4th-5th day. One observation deserves special comment: The group of Cercopitheci vaccinated with the smallest dose and clinically unaffected by the vaccination responded with slightly higher antibodies than those inoculated with large doses. The appearance of complement-fixing antibodies in titers >1:16 indicated that vaccination with the E.V. 51f produced a type of infection indistinguishable from that induced by slightly virulent P. pestis, possessing, colloquially called by Girard, "residual virulence". It is reasonable to suspect that the smaller dose caused less injuries than the larger doses.

(2) Vaccination of South African Cercopithecus aethiops pygerthrus. By request of the principal investigator, the South African Institute for Medical Research in Johannesburg provided facilities to conduct vaccination experiments on non-exportable South African green monkeys. Because of the devoted service and courtesy of Dr. M. Isaacson (Department of Bacteriology) and Mr. A. F. Hallett (Medical Ecology Centre) at the Institute, the essential findings of the partially completed studies have been made available. By their permission, the important observations which they will publish are here briefly detailed and analyzed.

Groups of 10 Cercopitheci, with both sexes fairly evenly distributed, weighing 4-6 kg each were inoculated subcutaneously with dilutions of the reconstituted freeze-dried living E.V. 76 51f. Vaccination fatalities occurred as follows:

		Total
Group I	Dose 1.009 x 10^8; death on day 4, 5, and 6 (2)	4
Group II	Dose 1.009 x 10^6; death on day 5 and 11	2
Group III	Dose 1.009 x 10^5; death on day 6, 7, 11, 22	4

TABLE 2

Clinical and Bacteriologic Response, Incidence of Vaccination Fatalities and Resistance to Virulent Plague Infection (106,000 P. pestis 195/P) of Cercopithecus aethiops (subsp. Kenya)

Animal Number and Dosage	Systemic Reactions			Day of Death*	Antibody Titers 28th day			Outcome of Challenge Infection
	Fever	W.B.C.	Blood Culture		HA	CF	MPI	
218 million								
CA 173	+	17,800	++	-	2048	4	4	Survived
CA 174	+	28,300	+++	7	-	-	-	-
CA 185	+	11,500	+++	20	-	-	-	-
CA 191	+	24,500	+++	15	-	-	-	-
2.18 million								
CA 176	+	66,900	++	7	-	-	-	-
CA 178	+	17,700	++	-	2048	>16	∞	Survived
CA 181	+	51,900	++	8	-	-	-	-
CA 190	+	25,600	++	6	-	-	-	-
218,000								
CA 175	+	16,700	000	-	2048	AC	∞	Survived
CA 179	-	44,900	+++	8	-	-	-	-
CA 183	+	16,000	+++	7	-	-	-	-
CA 187	+	18,000	++-	-	4096	2	3	Survived
2,180								
CA 177	-	21,700	++	-	512	8	5	Survived
CA 180	+	38,800	++	8	-	-	-	-
CA 182	-	17,900	+---	-	4096	>16	1	Survived
CA 189	-	23,000	-±-	28	-	-	-	-
218								
CA 184	-	16,000	---	-	1024	2	5	Survived
CA 186	-	10,900	---	-	8192	>16	1	Survived
CA 188	-	14,000	---	-	8192	8	4	Survived
CA 192	-	11,000	---	-	1024	4	∞	Survived
Control	-	-	0	-	0	0	-	Death, Day 5
Control	-	-	-	-	0	0	-	Death, Day 4

*Vaccinated subcutaneously with living attenuated P. pestis E.V. 76 (51f)

		Total
Group IV	Dose 1.009 x 10^3; death on day 8 and 28	2
Group V	Dose 101; death on day 9	1

According to sketchy reports, local and systemic reactions following vaccine inoculations were quite similar to those observed in Ethiopian and Kenya *Cercopitheci*. The erythemas and indurations at the site of inoculation disappeared within 4-5 days, but the regional (inguinal) lymph nodes enlarged in 2-3 days and persisted as marked, firm lymphadenopathies for up to 25 days. Fever was recorded irregularly in every animal; WBC were elevated up to 22,000 (even to 131,200 in one *Cercopithecus* shortly before its death on the 7th day). Blood cultures were obtained as early as the 2nd day and varied from a few to diffuse uncountable colonies per ml on blood agar surfaces. Even animals which survived had temporary blood-stream invasion. Gross and microscopic autopsy lesions were identical to those observed in other series: local inguinal lymphadenopathy, with little or no hemorrhage extending to iliac and para-aortic nodes; subcutaneous congestion; pleural and pericardial slightly blood-tinged fluids; splenic tumor; parenchymatous degeneration of kidney and liver, with microscopic necroses; adrenal hemorrhages; pulmonary congestion and edema. Late deaths were accompanied by emaciation and terminal enteritis (*Proteus mirabiles* and *P. rettgeri*). *P. pestis* was invariably isolated from the heart-blood and lymph nodes, thus confirming the post mortem diagnosis of bubonic-septicemic plague.

The antibody response of survivors was also similar to that observed in the Kenya series. The HA titers varied from 1:4 on the 2nd, 1:128 on the 8th, to 1:4096 and even 1:32,748 on the 28th day and the MPI of 10 animals varied from 1, 2, 3(4), 6 and 11 on the 28th day.

Since the animal quarters were urgently needed for other studies, the project had to be terminated by the 30th day; and since space in the isolation laboratory was limited, only 10 *Cercopitheci* -- two selected at random from each group -- were reserved for protection tests by experimental infection. They were inoculated subcutaneously on the 166th day (over 5 months) after vaccination with 8,770 highly virulent *P. pestis* (F357 Lesotho 1968 outbreak). Three of the challenged animals succumbed to septicemic plague:

Group I	737 - HA 1:32 WBC 23,000 (day 4) Blood culture negative (4); death on 8th day.
Group II	750 - HA 1:128 WBC 21,900 (day 8) Blood culture 150 cols/ml (8); death on 12th day.
Group IV	767 - HA 1:64 WBC 13,500 (day 4) Blood culture negative (4); death on 10th day.

Unvaccinated control 1 - WBC 31,300 (4) Blood culture 200 col/ml (4);
death on 6th day.

Unvaccinated control 2 - WBC 10,800 (2) Blood culture negative (2);
death on 7th day.

One of the two Cercopitheci in Group V developed a local suppurating axillary bubo as evidence of relative resistance, but recovered. Survivors of the experimental infection revealed no local reactions or lymphadenitis at the site of P. pestis inoculation and appeared well. Thus far only the HA readings on pre-infection sera are available; the two animals without apparent immunity had titers of 1:32 to 1:64 in comparison with 1:128 and 1:256 in others. All had declining titers which varied from 1:512 to 1:4096. No MPI readings immediately before infection are available, but it is reasonable to suspect that the high HA titers reflected significant mouse protective antibodies. No vaccine dose-immunity relationship was discernible. In every group, whether two or three animals were vaccinated with the highest (100 million organisms) to the lowest (100 cell dose), one was not protected. It is regrettable that unexpected circumstances limited experimental challenge infections to 10/37 or 27% of the survivors of the vaccination trial. Thus meaningful survival percentages could not be calculated. It is indeed surprising that the living, highly invasive, partially virulent E.V. 51f, which conferred absolute protection with as few as 218 viable organisms upon other Cercopithecus aethiops species for at least 52 days apparently failed in three South African species. As judged by the antibody response on the 28th day after vaccination, immunity was solid (MPI all below 5). The challenge infection was 1/15 of that used in preceding experiments on African green monkeys. Originally the experimental protocol called for determination of the susceptibility of the C. pygerthrus species, but since suitable isolation quarters were not available, only two nonvaccinated animals were infected. They succumbed, with anatomic and bacteriologic findings of acute septicemic plague, on the 6th and 7th day, respectively, or approximately at the same phase of the disease as Ethiopian Cercopitheci infected with 2 to 20,000 P. pestis 195/P. It may be tentatively assumed that the susceptibility of South African vervets is the same as that of the Ethiopian species. No experimental data are available to assess the immunizability of the C. pygerthrus species. Their immediate response to vaccination was excellent, as evidenced by the MPI (3) on the 28th day. But apparently their immunity was lost by the end of the 5th month. Since this finding is contrary to results with live bacterial vaccines, it is imperative that other investigators using Cercopitheci repeat the experiment and determine the duration of protection induced by a "safe" E.V. vaccine -- an aspect which will be discussed in subsequent paragraphs.

(3) Vaccination of langurs (Presbytis entellus) with E.V. 51f. The study of the effects of E.V. 51f on langurs was undertaken for the purpose of determining and measuring local and systemic reactions. In retrospect

it appears that valuable animals were needlessly sacrificed, since the outcome was predictable from preceding experiments on Cercopitheci. The essential observations are detailed in Table 3. The vaccine dose chosen represented the approximate number of viable P. pestis E.V. 51f to be tried on human subjects. It was soon discovered that the vaccine caused severe, painful, local inflammatory reactions which persisted for over two weeks; in fact nodules could be palpated as late as the 25th day. By contrast, local lymphadenopathy was not as marked when vaccination was accomplished by the cutaneous route. By the third day, the animals were lethargic and ate vegetables but no biscuits. All displayed fever, and 2/4 langurs which succumbed to vaccination on the 6th-7th day yielded positive blood cultures. Gross anatomical lesions at autopsy resembled those of acute bubonic-septicemic plague, with two significant exceptions: (a) little or no hemorrhage at the site of inoculation extending to the regional lymph nodes, which were enlarged, grayish or very slightly congested but not embedded in edema or hemorrhage. Thoracic, pericardial and abdominal cavities were free of fluid. Enlargement of the spleen and parenchymatous changes in the liver and kidneys, some hemorrhage in the adrenals, congestion of the lungs, with edema and dilatation of the ventricles and auricles of the heart were indistinguishable from the effects of virulent plague.

Early appearance of HA on the 8th and 16th days, with highly potent passive mouse protection antibodies (MPI - 00) including complement-fixing antibodies on the 28th day, attested to the solid protection conferred by vaccination of the langurs when they were infected on the 7th day. Local reactions due to the vaccine were definitive in the Cercopitheci included in the test series but were less marked than in the langurs. Lymphadenopathy was present but less prominent. One vervet (CA 206), clinically ill with a low-grade bacteremia, yielded the nonpigmented E.V. 51f in blood culture for at least 6 days. As in the langurs, the early appearance of antibodies and low MPI correlated exactly with the absolute protection proven by infection. The guinea pig-passaged E.V. 76 from Madagascar, concluded to be "safe" in tests on guinea pigs, apparently possesses a "residual virulence" which may prove lethal to Cercopithecus and langurs, since both are highly susceptible to virulent plague. Female Kenya Cercopitheci may be slightly less susceptible than males.

(4) Vaccination of langurs and macaques by the intradermal route using the bifurcated needle puncture technique. For years Soviet immunologists have advocated and practiced on human subjects the cutaneous technique of vaccination with live E.V. plague vaccines. After disinfection of the skin of the upper surface of the forearm with alcohol and drying with gauze, one drop of vaccine (6 billion organisms/ml) is applied in three areas at distances of 3 to 4 cm apart. A smallpox vaccination lancet is then used to apply one cruciform scratch at each of these points. The length of each incision is approximately 1 cm, and the distance between scratches should be 0.4 to 0.5 cm. Over a period of several seconds, the drops of vaccine are carefully rubbed into the incisions with the lancet.

TABLE 3 - PART 1

Clinical and Bacteriologic Response, Incidence of Vaccination Fatalities and Resistance to Virulent Plague Infection (307,000 P. pestis 195/P) of Langurs (Presbytis entellus) and Cercopithecus aethiops (subsp. Kenya)

E.V. 51f vaccine dose: 130 million viable, in 707 million total, number of cells, subcutaneously.

Animal Number	Local Reactions	Systemic Reactions	
		Fever (Day)	Blood Culture (Days)
SE 285	erythematous indurated area, 5 x 4 cm, 2nd day; 3.5 x 2 on 5th, painful inflammation; faded to 1 cm 16th day; lethargic, lymphadenopathy.	40 (2,5)	0 (1, 2, 4, 5, 8)
SE 294	purple induration 2 x 1.5, persistent lethargy, irregular anorexia 2-6th day.	40 (1,2)	+ (1) E, TNTC plate (5)
SE 319	raised purple indurated area, 6 x 4.5 cm; 4 x 3 cm 5th day; faded to 0.75 nodule. Lymphadenopathy; 7-8th day lethargy.	40 (2)	0 (5, 8)
SE 323	indurated purple area 6 x 2, persistent from 2nd day; lethargy and anorexia	40 (1, 2 & 3)	0 (E, 1); 24/ml (2), 188/ml (3), 136/ml (5)
CA 204 female	erythema & indurated area 2.5 x 1.5 cm 2nd day; 2 x 1.5 cm 5th day; induration, no erythema, faded to 0.5 (16th day), appears healthy and active	40 (1)	0 (5, 8)
CA 206 female	purple nodule 1.5 cm, hard reduced to 0.5 cm 5-16th day; hard nodule, active, erratic eating 6-9th day.	40 (4)	+ (E, 4), 4/ml (5), 4/ml (8)

Challenge Controls:
SE 293 - died 5-6th day
SE 288 - died 3-4th day
SE 317 - died 4-5th day

E = enriched in fluid media
TNTC = too numerous to count

TABLE 3 - PART 2

Animal Number	Antibody Titers - Day 8th HA	 8th CF	 16th HA	 16th CF	 47th HA	 47th CF	 47th MPI	Outcome of Challenge Infection on 70th Day After Vaccination
SE 285	512	-	2048	AC	2048	8	00	Survived. No local lesions.
SE 294	Vaccination death, 6th day							
SE 319	-	-	>8192	0	512	16	00	Survived. No local reaction.
SE 323	Vaccination death, 6-7th day							
CA 204	2048	0	2048	16	512	AC	3	Survived. No local reaction.
CA 206	512	-	512	2	512	2	3	Survived. No local reaction.

During the WHO Traveling Seminar on Plague Control in the USSR, the presentations and discussions at Stavropol emphasized the cutaneous method of live vaccine inoculation because of resulting *low reactivity*, adequate immunologic efficacy in tests on animals (guinea pigs), and the ease, simplicity, and practicality of the procedure. On the other hand, *intracutaneous* (intradermal) inoculation makes it possible to dose vaccines accurately and yields the best results in testing for protection against plague. In fact it was stated that intracutaneous vaccination affords protection against both the bubonic and pulmonary forms of plague. Comparative experiments were cited in which guinea pigs were vaccinated by the intracutaneous and subcutaneous methods with doses of live vaccine as small (!) as 20,000 bacteria. In one test of this kind, all the animals vaccinated intracutaneously survived after infection by 400 lethal doses of virulent culture 30 days later. Only 80% of those vaccinated subcutaneously survived. However, it was emphasized that a correctly administered intracutaneous inoculation, employing an active vaccine strain, nearly always leads to a "characteristic" local reaction. In typical cases, reaction develops rapidly: 4 to 5 hours after the intracutaneous administration of *1.5 million* bacteria in 0.2 ml of saline, a dark-red papule 0.5-0.6 cm in diameter appears at the spot of injection. In approximately 24 to 30 hours, the papule becomes a vesicule, which is then transformed into a bluish-purple pustule. As these symptoms proceed, the skin around the point of infection reddens markedly and becomes swollen, indurated and hot to the touch. The area of reddening extends to 15 x 15 cm, while pain of varying intensity results from moving the arm. After 3 or 4 days, the pustule may break open and release a sanguinolent, purulent exudate yielding colonies of the *P. pestis* vaccine strain upon culture. Frequently the pustule will not open, but will dry up gradually, forming a scab which falls off 6 to 8 days later, leaving a slight scar on the skin. The local reactions described occur in an overall period of 8 to 10 days. Lymphangitis and lymphadenopathy of the forearm and axilla are common. General symptoms are usually evident on the 3rd to 4th day following inoculation. Temperature rises and other generalized reactive symptoms of intracutaneous inoculation in no way differ from those observed after subcutaneous inoculation, either in their intensity or frequency. Mainly they depend upon bodily characteristics of the individual human subject.

These reports made it essential to study the efficacy of the intracutaneous injection technique with live plague vaccines in highly susceptible subhuman primates. The planning of these experiments was guided by the conviction that the highly "active" E.V. 51f would prove harmless and solidly immunize langurs and macaques. With the introduction of the bifurcated puncture needle, a simple and fairly quantitative vaccination technique became available. Preliminary intracutaneous vaccination trials on guinea pigs fully established the marked invasiveness of the attenuated E.V. 51f. With the use of the multiple pressure method, scarification with a tuberculin chisel, intracutaneous inoculation of dilutions by needle, or the one-puncture technique employing the bifurcated needle, it was established that approximately 8000 viable attenuated P. pestis E. V. 51f confers 100% solid immunity with significant antibody levels, while 800 protected 80%, and 80 were effective in 70% in groups of 10 guinea pigs. The lowest guinea pig protective dose of the freeze-dried vaccine administered subcutaneously is 3000. For subhuman primates on a pro-weight basis it was estimated at 30,000. The actual experiment demonstrated that the estimate was erroneous. For langurs probably the lowest protective dose is less than 300.

The original purpose of the experiment using the intradermal puncture technique was (a) to gauge the intensity of the local reactions anticipated from previous experiments on langurs and vervets; (b) to compare the reactions in macaques; and (c) to determine in a few animals the approximate dose required to give specific protection. Well-conditioned langurs weighing from 5 to 8 kg each and Macaca mulatta (3-5 kg) were shaved on the chest over the xiphoid bone and the skin was cleansed with alcohol. New sterile bifurcated needles were dipped into the reconstituted diluted vaccine dilutions contained in small Petri dishes at a level to which only the space between the two forks was filled with fluid. The needle was gently pushed into the skin and rapidly withdrawn without extracting blood. Carefully prepared cultures of the vaccine suspension retained in the bifurcations and the needles indicated that in trial test cultures approximately 842,000, 84,000 or 8000 viable E.V. 51f were delivered to the puncture wound. Parallel tests on guinea pigs revealed that in technique with the needle charged with 8000 organisms 9/9 guinea pigs were protected. Although separate needles were used for each animal, it was subsequently discovered that their points were of variable sharpness. Thus a single puncture may not deliver the desired vaccine dosage. As usual, plague studies on langurs resulted in unexpected findings, as detailed in Table 4: (1) Local reactions in successfully vaccinated animals were manifest as pinhead-sized, pink-to-deep-red nodules apparent by the 24th hour, subsiding to faintly visible needle-points by the 5th day. At autopsy, neither the skin nor subcutaneous tissues revealed inflammatory reactions at the site of puncture. (2) Within 5 days, the axillary lymph nodes (either the right or left) were palpable and attained the size of large peas or even small peanuts, persisting for more than 20 days in the only survivors. (3) Beginning on the 2nd day in one langur (SE 322) vaccinated with the 800,000 dose, and in three others on the 5th to 9th days, the body temperature rose to 40^{0} or above. (4) After the 5th day, successfully vaccinated monkeys were clinically ill and decidedly less active, consumed less food, and finally exhibited complete anorexia; all developed diarrhea. (5) Daily quantitative blood cultures yielded blood-plate surface colonies of typical E.V. 51f varying from 24 to >200/ml. (6) Biopsy aspirates of the enlarged axillary lymph nodes on the 12th day invariably produced heavy growth on blood plates, but only one revealed typical plague bacilli in Giemsa-stained smears obtained in order to study the types of monocytes in the nodes. (7) As early as the 8th day after vaccination, hemagglutinins appeared in titers from

TABLE 4 - PART 1

Clinical and Bacteriologic Response, Incidence of Fatalities Observed in Langurs (Presbytis entellus) and Macaca mulatta Intradermally Vaccinated With E.V. 51f by Single Punctures with Bifurcated Needles.

Animal Number	Approximate Vaccine Dosage	Local Reactions	
		Site of Puncture (Day)	Lymph Nodes (Day)
SE 345	842,550	red, small nodules (2), needle point (5)	R axillary, pea size (8)
SE 322	"	small pink nodule (2), needle point with scab	L axillary, peanut (5), cluster (9), soft
SE 332	84,255	red pinpoint (2), needle point (5, 8)	L axillary, palpable (5, 8, 9), almond (12)
SE 321	"	pinpoint (2), needle point (5)	R axillary, peanut; L, palpable
SE 339	8,425	pinpoint (2) to needle point	0
SE 343	"	indistinct pigmentation, erythema 4 x 5 cm	L small cherry (9)
MM 5804	842,550	needlepoint	none palpable
MM 5800	"	needlepoint	"
MM 5378	84,255	needlepoint	"
MM 5444	"	needlepoint	"

TABLE 4 - PART 2

Animal Number	Systemic Reactions (Day) Highest Body Temperature	Blood Culture	Biopsy Culture
SE 345	40.2 (8)	0 (2), 0 (5), 24/ml (8)	R axillary, large pea, +++ (12)
SE 322	40.1 (5, 8, 9) 40.7 (7)	0 (2), 26/ml (5), 146/ml (7), 32/ml (8), 30/ml (9)	L axillary, +++ (12)
SE 332	40.2 (9)	0 (2, 5, 8) TNTC (9)	--
SE 321	40 (5, 9) 40.3 (7)	0 (2), >200/ml (5), 136/ml (8), 36/ml (9)	--
SE 339	40.1 (6)	0 (2. 8)	--
SE 343	40.3 (7, 8)	0 (2), 4/ml (5), 66/ml (7), 14/ml (8), N.D. (9)	smear +
MM 5804	39 (8)	NE (2, 5, 8)	--
MM 5800	39.1 (5)	NE (2, 5, 8)	--
MM 5378	39.2 (2,5)	NE (2, 5, 8)	--
MM 5444	39.6 (8)	NE (2, 5, 8)	--

NE = negative enrichment
TNTC = too numerous to count
N.D. - not done

TABLE 4 - PART 3

Animal Number	Vaccination deaths, Day-Autopsy
SE 345	--
SE 322	17. Acute plague without classical buboes, no edema or hemorrhages; abdominal exudate; very large spleen; liver necroses; nephritis; marginal pulmonary consolidation; R. cardiac dilatation; enteritis.
SE 332	12-13. Acute plague, atypical buboes; metastatic pneumonia areas; lung edema, hemorrhagic enteritis.
SE 321	16. Acute plague; hemorrhage respiratory and intestinal tract, granuloma-like lesions, lymph nodes.
SE 339	--
SE 343	13. Acute plague, no edema or hemorrhages; splenic tumor, pulmonary hypostasis, enteritis.
MM 5804	--
MM 5800	--
MM 5378	--
MM 5444	--

TABLE 4 - PART 4

Animal Number	Culture			29 Days Post Inoculation Antibody Titers			Response to Challenge*
	Heartblood	Spleen	Lung	HA	CF	MPI	
SE 345	-	-	-	8192	32	1	solid protection
SE 322	+	+++	+++	(2048-8 days)			
SE 332	+	+++	++	0 (8)			
SE 321	+	+++	++	512 (8)			
SE 339				0	0	16	acute bubonic plague, day 4
SE 343	++	+++	+	128 (8)			
MM 5804				2048	0	2	
MM 5800				4096	0	5	
MM 5378				4	0	20	
MM 5444				256	0	2	

*Challenge with 342,000 virulent _P. pestis_ on 161st day.

1:64 to 1:2048. (8) Between the 12th to 17th day, four of five successfully vaccinated animals succumbed, with unique gross anatomical lesions of acute plague complicated by enteritis of varying intensity but revealing no evidence of the pleural and pericardial exudate common in virulent infections. Lesions in the enlarged axillary lymph nodes draining the vaccination area were particularly striking. They were firm, grayish, granulomatous, even necrotic, without hemorrhages except in those biopsied and not surrounded by areas of edema. The spleen was always enlarged and pulpy; in SE 322, it was exceptionally large, accompanied by the necrosis-studded liver commonly seen in African green monkeys subjected to attenuated plague inoculation. With one exception (SE 342), metastatic pneumonic areas of consolidation of varying extent were present in the poorly collapsed lungs. Right cardiac dilatation due to partially coagulated blood was observed in every animal. This manifestation of toxemia was complicated by hemorrhagic gastoenteritis. In SE 332, bleeding from the nasal passages and upper respiratory tract involved the right anterior lobe. Parenchymatous changes in the kidney -- nephritis, urine with high protein content -- and adrenal hemorrhage were similar to those seen in virulent infections. (9) Bacteriological examinations using four different culture media (including Gentian violet, McConkey agar plates) demonstrates *P. pestis* 51f in all organs and tissues, generally mixed with *E. coli* and *Klebsiella*. Even the heart-blood, liver, spleen and axillary lymph nodes, removed carefully from the carcass of a langur (SE 322) sacrificed while moribund, yielded isolated colonies of *E. coli* and *Klebsiella*, aside from heavy growth of *P. pestis*. (10) Of the two langurs surviving vaccination, one (SE 339) remained well but developed no antibodies and finally succumbed to virulent infection; the other (SE 345) recovered from a large-dose puncture vaccination and proved solidly immune when challenged 167 days (24 weeks) after inoculation with 343,000 virulent *P. pestis* 195/P.

Comparing the response of *Macaca mulatta* to E.V. 51f intradermally administered with a single bifurcated needle puncture to that observed in langurs, the former's low susceptibility to plague was again strikingly apparent. No local cutaneous reactions, enlargement of regional lymph nodes, nor systemic reactions could be detected. The animals remained well and repeated blood cultures continued to be sterile. Nevertheless, the antibody response was vigorous in three *Macaca* with MPI of 5 or below. The single puncture apparently failed to infect MM 5378. The immunity of the successfully vaccinated has not yet been challenged by infection.

Based on this single pilot experiment on macaques, the E.V. 51f isolate would appear to be a safe, highly efficient vaccine producing no local or systemic reactions. An opposite interpretation, however, must be derived from analysis of the clinical test on langurs. Although local reactions produced by the intradermal puncture technique were slight, it was disconcerting to note that in this highly susceptible species, the E.V. 51f proved highly invasive, causing slowly developing generalized infections, reflected by positive blood cultures beginning on the 5th day and rapid enlargement of the regional lymph nodes. The pathophysiology is not clearly discernible, but it is likely that a progressive toxemia accompanied by severe injuries to the parenchymatous organs finally led to intestinal disturbances and vascular damage. Terminal septicemia was complicated by invasion of the intestinal flora. The "residual virulence", which, according to numerous experiments on guinea pigs, leads to only temporary invasion of the blood-stream or lungs, was pronounced in langurs. The blood-stream was constantly seeded with virulent members of the E.V. 51f population, leading to consequent secondary pneumonia in the lungs of langurs dying on the 12th to 17th days after vaccination -- a sequence of events compatible with the usual course of protracted infections with virulent P. pestis. Further studies are required to determine and explain the molecular microbiologic mechanism responsible for a phenomenon not clearly understood from experiments with guinea pigs or macaques. In this connection, it is pertinent to comment on a recently discovered correlation: In one experiment, the E.V. 76 isolates obtained after passage through guinea pigs treated with iron sulfate, highly pathogenic for langurs, caused lethal infections upon intravenous inoculation in mice. For example, E.V. 51f in dosages of 20 viable organisms proved lethal for 10%, while 2,000 caused an 80% mortality in the inoculated mice. If future studies with other attenuated P. pestis confirm these observations, this inexpensive pathogenicity test may screen E.V. 76 or other attenuated live plague vaccines permitting their categorization as "unsafe" and "safe" preparations. This aspect will be the subject of thorough inquiry.

(5) Vaccination studies with E.V. 51f on wild rodents -- the multimammate mouse (Mastomys or Praomys natalensis) and the Kurdistan gerbil (Meriones tristrami). In the search for less expensive but equally susceptible mammals other than subhuman primates, it was recalled that Murray and Davis (South African J. Med. Sci. 28:58, 1963) had reported Praomys natalensis to be a standard test animal for experimental work on plague. Amies (Brit. J. Exp. Path. 32:259-273, 1951) concluded that this mouse may be employed advantageously to assay plague vaccine antigens intended for human prophylactic use. In collaboration with Mr. A. F. Hallett at the Medical Ecology Centre, the State Department of Health, and the South African Institute for Medical Research, a series of vaccination studies using E.V. 51f were conducted on these animals (weighing 30 gm each). With his permission the preliminary results of this incomplete experiment are summarized in Table 5.

Interpretations of the results are those of the principal investigator. The freeze-dried E.V. 51f vaccine which was sent to South Africa was reconstituted and inoculated subcutaneously in dilutions causing a 30% fatality rate in Group I (170 million organisms) and a 10% mortality (1.7 million). With three weaker dilutions which were all well tolerated, only animals in Group III responded with HA titers (1:8 to 1:512). A single animal in Group IV had an HA of 1:16 and survived the challenge infection. A vaccine dose of 1,700 proved non-immunogenic, as reflected

TABLE 5

Incidence of Vaccination Fatalities Produced by E.V. 51f and Immunity of Survivors to Virulent Plague Among Praomys (Mastomys) natalensis

Group and Vaccine Dosage	Day Death Following Vaccination Autopsy Findings	Response to challenge on 34th day, 6,000 P. pestis (F357 Lesotho 1968)
I. 1.7×10^8 170 million	3. D/T = 3/10 = 30% mortality. Acute plague with hemorrhagic edema, pleural exudate necroses. Survivors HA: 64-1024	S/T = 4/6 = 66% mortality. Average day of death: 11. Typical pathology, *P. pestis* +++. Survivors: residual pulmonary lesions on 71st day.
II. 1.7×10^6 1.7 million	5. D/T = 1/10 = 10% mortality. Autopsy as in Group I.	S/T = 3/5 = 60% mortality. Average day of death: 6. Typical pathology, *P. pestis* +++. Survivors: 2 residual pulmonary lesions.
III. 1.7×10^5 170,000	D/T = 0/10 = 0 mortality.	S/T = 4/6 = 66% mortality. Average day of death: 5.5. Typical pathology, *P. pestis* +++. Survivors: 2 residual pulmonary lesions.
IV. 1.7×10^3 17,000	D/T = 0/10 = 0 mortality	S/T = 1/6 = 17% mortality. Average day of death: 4.4.
V. 1.7×10^2 1,700	D/T = 0/10 = 0 mortality	S/T = 0/6 = 100% mortality. Average day of death 4.1.
Controls	-	S/T = 0/10 = 100% mortality. Average day of death: 4.6.

+++ = septicemia
D/T = Deaths/Total
S/T = Survivors/Total

by serum antibodies. Fatality rates following infection with avirulent wild P. pestis from South Africa are roughly related to the vaccine dose. Resistance, however, is definitely relative: 7/10 when sacrificed on the 71st day after challenge presented residual lymphadenopathy and the pulmonary lesions of resolving plague at autopsy. One Praomys of each group was revaccinated intraperitoneally with 1,700,000 viable E.V. 51f on the 71st day after primary vaccination. Bleeding accidents reduced the number to three. Two (one each, Groups II and V) survived, while the one from Group I succumbed to plague on the 17th day. Survival after revaccination of animals in a group which exhibited no resistance after primary inoculation may be either coincidental or significant. The HA titers of the survivors (1:512 to 1:16,384) were contrasted to the HA (1:32) recorded for the Praomys, which, despite revaccination, died on the 17th day.

In a second trial, 50 Praomys in five groups of 10 were vaccinated with dilutions of the reconstituted E.V. 51f by means of one-puncture technique with the bifurcated needle, with no local reactions nor mortalities. Twenty-nine days after vaccination, the rodents were challenged with 8,420 P. pestis (F357) Lesotho. In Group I, intradermally vaccinated with approximately 466,900 viable organisms, 6/10 or 60% survived; Group II animals were inoculated with 46,690 organisms, and 3/10 or 30% survived. In Groups III (4,669), IV (467), and V (47), there were no survivors (0/10). Available records indicate that no local reactions were observed. As expected from the results of the first experiment, even if the total dose of 466,000 viable organisms was inoculated, no vaccination deaths occurred, and the survival percentages tallied closely with those of the first test. Whether the immunity conferred by the vaccine was merely relative or absolute will not be ascertained until the survivors have been examined by autopsy. The second experiment supplemented the first and confirmed the impression that Praomys, highly susceptible to plague, are difficult to immunize with the attenuated living E.V. 51f. According to Grasset (1942), Praomys tolerated the inoculation of 400 million of the live plague vaccine (K/120) used for mass vaccination in South Africa. Whether the rodents were proven protected was not recorded.

These pilot experiments with the attenuated P. pestis E.V. 51f in a South African wild rodent species indicate that dosage definitely influences the outcome of vaccination. With doses of 1 million and above, fatalities may be anticipated. Regardless of the method of vaccination, doses of 500,000 to 1.7 million confer approximately 60% relative protection.

During the International Congress on Tropical Medicine and Malaria held at Teheran in September 1968, Dr. M. Bahmanyar kindly arranged for Dr. Y. Karimi of the Institut Pasteur de l'Iran, Teheran, to conduct a vaccination experiment on the susceptible gerbil Meriones tristrami. A recently available brief report on the test indicates that wild gerbils tolerated subcutaneous inoculations of 507 to 507 million organisms of the E.V. 51f vaccine with no local and systemic reactions. HA antibodies were demonstrated in the sera of animals inoculated with the largest dose (1:64 to 1:16,348), but with dilutions 5 million, 500,000 and 5,000, HA antibodies were inconsistent, which was not the case with 500 organisms. This exception implies that the immunity mechanisms of Meriones are apparently different from those of the South African Praomys natalensis. A smaller vaccine inoculation (100 organisms) stimulated the appearance of HA (1:256 to 1:4096) and even caused one vaccination (?) fatality. Only 8/19 or 44% of the vaccinated wild rodents responded with antibodies. Correspondingly, they were not protected when infected with 750,000 P. pestis PKR 684. Only one (?) rodent in Group I (inoculated with 500 million E.V. 51f) exhibited some degree of protection; one survived, while death was delayed in four (5th, 7th, 16th and 19th days), as compared to the mortality of 23 controls (3-4 days). Group II demonstrated equally delayed deaths (7th day). The rodents from burrows in Iran originating from a heterogenic population proved just as resistant to the pathogenic effects of E.V. 51f as guinea pigs. But why they are incapable of developing protection with a vaccine proven highly efficacious in other mammals remains intriguingly unknown. The solution of the problems involved may contribute to a clearer understanding of wild rodents' variable resistance and mechanism of immunity to natural plague infections in endemic areas. The few vaccine studies on Praomys and Meriones tristrami provided no comparable substitutes for subhuman primates, but they did furnish additional evidence that the high susceptibility to plague in Praomys is not simultaneously accompanied by an efficient immunity mechanism. Does the rampant multiplication of the "virulent" component of the vaccine, with the resulting release of endotoxin, damage the lymphoreticular tissues? This possibility has not received enough attention in recent discussions involving protective immunity with live plague vaccines.

(6) Immunogenicity of E.V. 51f as a killed vaccine. Several old reports dealing with live plague vaccine stress that when the strains are killed either by chemicals or by heat, they lose their protective properties. These statements, however, are contradicted by the fact that a variety of killed vaccines of considerable immunogenicity have been manufactured by Rowland (1912) and recently (1967) by Smith and Packmann from attenuated P. pestis.

A killed plague vaccine was readily prepared from a suspension obtained by rehydrating P. pestis E.V. 51 from 15 vials of freeze-dried vaccine, which was treated in distilled water with formalin and maintained for several days at ±4^{0}C. When it proved sterile, it was diluted to a concentration of 4000 million P. pestis per ml containing 0.05% formalin and 0.5% phenol. Series of guinea pigs were inoculated subcutaneously in single doses with 0.5 ml of 2 billion or 2 million of the aqueous suspension, with or without a second inoculation of a 1:10 dilution of the basic vaccine. Other groups received the same vaccine dilution emulsified in incomplete Freund's adjuvant. The aqueous killed E.V. 51f vaccine in a single dose of 2 billion or 2 million conferred no protection. Of a group basically inoculated with 2 billion and given single booster doses on the 30th day, 10-20% were protected. A single dose of vaccine in adjuvant protected 80% of the guinea pigs against infection with 870,000 virulent P. pestis 2 months after primary inoculation. Only 10% protection was attained with 2 million killed vaccine in adjuvant. The protective properties of the killed E.V. 51f vaccine are nearly identical to those reported for the U.S.P. vaccine. The killing process deprived the P. pestis of its invasiveness and multiplication capacity but did not alter its antigenic structure.

(7) Vaccination studies with a transplant from the original live attenuated P. pestis E.V. 76 (stored in Paris). The disconcerting experiences just reported involving vaccination experiments using subhuman primates and wild rodents with descendants (Saigon, Madagascar) of the E.V. 76 isolate logically required testing a transplant of the original culture. Through the courtesy of Dr. G. Girard, a subculture of his isolate E.V. 76 maintained in regular passage on agar was obtained in December 1967. Protective efficacy tested in guinea pigs early in 1969 was still of a high order, as illustrated by the results recorded in Tables 6 and 7.

The number of viable organisms capable of protecting Hartley guinea pigs is expressed by the PD_{50} of 40 by the intracutaneous route and by the PD_{50} of 33 by the subcutaneous route. Even in a dose of 100,000 organisms, the E.V. 76 (Paris) is nonpathogenic for mice inoculated by the intravenous route. The PD_{50} determined for mice by the intraperitoneal route was 38 and by the subcutaneous, 2800. The immunogenic and "granuloma"-forming properties in guinea pigs, specified for the original isolate in 1932, were completely retained during its maintenance in the Institut Pasteur for over 37 years. All the biochemical nutritional marker determinants -- F_1+, pigment negative, CA dependence +, uracil independence, Pesticin I, Pesticin II+, Fibrinolysis -- originally recognized by Burrows and confirmed by Surgalla and others were readily demonstrable.

The transplant E.V. 76 (Paris), grown on E medium agar at 28°C in the form of a saline suspension, was used in a vaccination experiment involving 4 langurs, 6 African vervets, and 4 macaques intradermally (left femur) inoculated with 26,800,000 viable organisms in 0.1 ml (Table 8). The experiment is still in progress, but the test with langurs has been completed, with the exception of correlating antibodies and resistance to infection. Regardless of weight (8-15 kg) and sex, local reactions in the form of erythematous indurated areas (2 x 2 cm) present by the 48th hour after inoculation began to fade by the 5th day, but induration was still marked on the 8th day and was palpable as a nodule on the 16th day and a hairless spot (0.9 x 0.9 cm) by the 27th day. By the 48th hour, left inguinal lymph nodes varying in size from 2.5 x 1.2 cm were already painful and firm but movable. Langur 325, severely ill for 3 days, also developed lymphadenopathy in the right inguinal nodes, which continued to enlarge until the 16th day, and in one large langur (SE 309) measured

TABLE 6

Protective Efficacy of E.V. 76 (Paris 1967) in Guinea Pigs

E. V. 76 (Paris 1967) intracutaneous or subcutaneous vaccination with 48 hour broth culture (HIV).

Antibody Titers

Intracutaneous Vaccination						Subcutaneous Vaccination		
MPI	HA	CF	S/T	Viable	S/T	MPI	HA	CF
3	2048	8	6/6	100,000	6/6	3	1024	4
4	1024	4				21	16	0
5	1024	4				4	1024	8
6	512	4						
18	64	0	6/6	10,000	6/6	10	512	2
9	1024	4				1	512	8
7	1024	2				6	1024	4
11	2048	2				17	64	0
			5/6	1,000	5/6			
19	0	0	5/6	100	5/6	21	8	0
3	1024	4				10	0	0
7	256	0				2	1024	4
10	512	2				18	0	0
12	0	0	1/6	10	1/6	17	0	0
14	0	0				16	0	0
10	0	0				19	0	0
14	1024	8				18	0	0
12	0	0	0/6	1	0/6	16	0	0
16	0	0				14	0	0
13	0	0				17	0	0
14	0	0				19	0	0

PD_{50} - 40 viable

PD_{50} - 33 viable

Mice PD_{50} - IP 38
Subc. 2,800

S/T = Survivors/Total

TABLE 7

Protective Efficacy in Guinea Pigs of E.V. 76 (Paris 1967) Grown at 28° for 48 hours

Estimate		Actual		
Total	Viable	Viable	S/T	HA of bleeders
130,000,000	40,000,000	26,300,000	6/6	64, 128, 128, 256
13,000,000	4,000,000	2,680,000	6/6	0, 4, 32, 4096
1,300,000	400,000	268,000	6/6	0, 0, 16, 64
130,000	40,000	26,800	6/6	32, 64, 256, 1024
13,000	4,000	2,680	5/6	0, 0, 64, 128
1,300	400	268	0/6	0, 0, 0, 1024
130	40	26	2/6	0, 0, 0, 0
13	4	2.6	0/6	0, 0, 0, 0
Control 1,230,000			0/6	8.3 average day of death

PD_{50} viable organisms 800

TABLE 8 - PART 1

Clinical and Pathological Effect of Agar Grown (28°C) P. pestis E.V. 76 (Paris 1967) Following Intradermal Inoculation of 26.8 Million Viable Organisms in 3 Species of Subhuman Primates.

		Local Reaction	
Species Number	Weight (kg)	Site of inoculation 2-10 days	Lymph Nodes[1] (Day)
SE 291	8.0	nodule, indurated area 2 x 2 cm	L. 3 x 2 to 4.2 x 1.8 (8)
SE 302	15.4	erythema, indurated area 2 x 2	L. 1.1 x 3.0 to 4.0 x 2.4 (8)
SE 309	15.3	erythema, indurated area 2.8 x 2.8, eschar	L. 2.5 x 1.0 to 5 x 3.6 (16)
SE 325	9.0	erythema, indurated area 2.7 x 2.7	L. 3 x 1 to 4 x 2 (12). R. 2 x 1.3 (8)
CA 231	5.5	erythema, 1.4 x 1.4 cm	L. 2.8 x 1.5 to 3.4 x 2.1
CA 232	4.9	pale nodule 2 x 2 cm	L. 2 x 2 to 2.6 x 1.7
CA 235	5.0	pale nodule 1.5 cm eschar	L. 1.5 x 1.1 to 2.9 x 1.9
CA 236	5.2	pale nodule 1.1 cm eschar	L. 1.5 x 1.1 to 2.4 x 1.4
CA 241	5.1	pale nodule 1.1 cm eschar, desquamation of skin	L. 0.5 x 0.5 to 2 x 3
CA 250	5.0	pale nodule 0.5 cm and induration, eschar	L. 2.8 x 2.5 to 3.1 x 2.1
MM 5258	5.9	pale nodule 0.8 x 1	L. 2.7 x 2.0 (5 day)
MM 5810	4.2	nodule 0.8	L. 1.4 + dysentery
MM 5891	4.2	pink nodule 0.5 x 0.5	L. 2 x 1 to 2.9 x 1.6 (5 day). 1.2 x 1.5 (7 day). R. 1.5 x 1.5 (7 day).
MM 5910	3.8	pink nodule 0.5 x 0.5	L. 2 x 1 to 2.9 x 1.6 (5). 1.2 x 1.5 (7 day) R. 1.5 x 1.5 (7 day)

[1] Left inguinal, Right inguinal lymph node, figures centimeter (Day) from 3 to 8-12 days.

TABLE 8 - PART 2

Species Number	Systemic Reaction (Day)			Biopsy Culture[3] (Day)
	Temperature	Blood Culture[2]	Leucocytosis	
SE 291	38.5 - 40.3 sl. diarrhea	2c (5)	+ 27,000 (8)	NT
SE 302	38.6 - 40.1	0	± 13,100 (8)	++ (5)
SE 309	38.6 - 39.7	0	- 9,300 (0)	++++ (5)
SE 325	37.7 - 40.2 sl. diarrhea	16c (4&8) E (5)	± 18,600 (8)	++ (5)
CA 231	39 - 40.3	C (5)	+ 30,400 (5)	++++ (5)
CA 232	38.7 - 40.2	E (2)	+ 23,300 (5)	++ (5)
CA 235	39 - 39.8	0	+ 22,400 (5)	10 col. (8)
CA 236	38.7 - 40.3	0	+ 21,600 (5)	NT
CA 241	38.7 - 40.5	C (5)	+ 34,000 (5)	++ (5)
CA 250	38.9 - 40.1	0	+ 32,700 (5)	100 col. (5)
MM 5258	38.8 - 39.3 diarrhea	0	- 12,500 (5)	NT
MM 5810	38.7 - 39.6 diarrhea	C & E (5)	- 17,200 (2)	NT
MM 5891	38.9 - 39.7	0	- 17,000 (0)	0
MM 5910	38.7 - 39.1	0	- 23,000 (0)	NT

[2] C - surface blood agar plate; E - broth culture 0.5 ml

[3] moderate = ++; heavy confluent = ++++; col. = colonies; NT = not tested

TABLE 8 - PART 3

Species	Antibody Titers					Day of Infection - 42nd[4]
	8th day		27th day			
Number	HA	CF	HA	CF	MPI	Pathology to Plague Infection
SE 291	256	0	4096	4		no local reaction, 5th day site of injection
SE 302	1024	0	1024	32		" " "
SE 309	512	0	1024	128		small erythematous nodule
SE 325	-	-	512	16		needle point at site of injection
CA 231	64	0	512	4		To be challenged
CA 232	8192	0	512	4		"
CA 235	512	0	256	8		"
CA 236	1024	0	4096	32		"
CA 241	256	0	1024	32		"
CA 250	512	0	64	8		"
MM 5258	32	0	0	0		Died 49th day, intercurrent Streptococcus infection
MM 5810	Died 8th day, *P. pestis* septicemia, Klebsiella (lung).					
MM 5891	32	0	128	32		
MM 5910	32	0	64	16		

[4] (with 343,000 *P. pestis* 195/P)

5 x 3 cm. On the 27th day, the nodes had only moderately receded (4 x 2 cm), but were softer in consistency. Reactions were of the same magnitude as those observed in the series of langurs (Table 2) vaccinated with small doses of the more invasive E.V. 51f. Painful local signs were accompanied by systemic reactions in the form of 1-2^{0} rises in body temperature, moderate leucocytosis, anorexia, loss of aggressiveness for at least 5 days, and diarrhea in the smaller animals. The recovery period extended over 8 days. Quantitative blood cultures yielded from 2 to 16 colonies in the two young langurs on the 2nd, 4th and 8th day. On the 5th day, biopsies of the lymph nodes of three animals revealed moderate to abundant growth of the vaccine strain, although microscopically, careful search of the smears revealing arrays of monocytes cell and lymphocytes proved nonproductive.

By the 8th day, HA antibody titers were already significantly high; and by the 27th day after vaccination, the appearance of complement-fixing antibodies in titers up to 1:128, together with high HA levels, conclusively contradicted reports that live attenuated plague vaccines fail to induce peripheral antibodies.

On the 42nd day after vaccination, immunity tests by subcutaneous (xiphoid region) challenge with 343,000 P. pestis 195/P revealed absolute protection, illustrated by the absence of local reactions in the skin and regional axillary lymph nodes. The nonvaccinated controls which died on the 4th day, as well as other langurs used in an experiment to evaluate the immunogenic efficacy of Fraction 1, displayed extensive hemorrhagic necrotizing dermatitis and lymphadenitis.

In Kenya vervet monkeys, local skin reactions were less marked than in the langurs: pale nodules surrounded by faintly reddish, slightly indurated areas were replaced by eschars or scaly desquamation of the readily folding skin by the 8th day. Enlargement of the left inguinal lymph nodes was moderate in comparison to that seen in langurs; evolution to prominent nodes from the 5th to 16th day, receding to 1.8 cm in size by the 27th day, differed from such reactions in the langurs only in intensity. Four of the six vervets exhibited temporary rises in temperature, which may have been the result of stress during capture, particularly in those which remained aggressive in the course of daily examinations. All were eating well but revealed leucocytosis, and three animals bled on the day they had body temperatures above 40^{0} yielded a few colonies on blood plates or by enrichment in broth culture on the 2nd or 5th day. Lymph node aspirates obtained by biopsy on the 5th day yielded the same results as those recorded for langurs. A large node in CA 235 when punctured disclosed only a few P. pestis (Paris). Antibody response was similar to that observed in langurs, but the protection conferred was relative; 2/6 vervets developed pustules at the site of infection (see page 9).

Local reactions in a group of macaques (weighing 3.8-5.9 kg each), previously used in malaria experiments and not in prime condition, were confined to pinhead-sized, pink nodules which were reduced to hairless areas by the 8th day. Regional adenopathy in the form of firm but movable nodes (maximum 2.9 x 2.0 cm) was similar to that observed in this species of subhuman primate vaccinated with the Fraction negative M23. Biopsy of the large left inguinal node at the time when the right node became palpable proved fruitless. No rises in temperature nor leucocytosis were recorded. Nonspecific diarrhea developed in two animals; one yielded a positive blood culture two days before death. Autopsy findings were atypical for plague and apparently complicated by nonspecific gastroenteritis and a chronic pneumonic abscess due to _Klebsiella_ sp. Nonetheless E.V. 76 (Paris) was isolated from the heart-blood, left and right inguinal lymph nodes, left enlarged iliac lymph node, spleen, liver and bile. Intercurrent _Streptococcus_ infection eliminated macaque MM 5810 on the 49th day while it was being held for further tests in quarters with other animals under inadequate supervision.

Serologic response was less vigorous than in langurs and vervet monkeys; one animal (MM 5288) had no HA and CF antibodies. The outcome of the immunity tests which will be made 3 months after basic vaccination is awaited with great interest. The invasiveness and residual virulence of the E.V. 76 (Paris), combined with a high immunity rate but accompanied by definite local and systemic side effects (probably essential for an efficacious live plague vaccine) in langurs and vervets, give rise to the pertinent question: Is it really "harmless" for man? In comparison with E.V. 51f, which caused lethal infection in highly susceptible subhuman primates with much smaller doses (Table 3, Part II), it appears relatively "harmless". However, only clinical trials with carefully supervised human subjects, using the intradermal single- or double-puncture vaccination technique with the bifurcated needle, will provide the answer to the important question: Is E.V. 76 (Paris) a safe and protective live plague vaccine? In one experiment on 6 African green monkeys the protection was definitely relative. _P. pestis_ 195/P persisted for 9 days in the fistular discharge of pustules at the site of infection of two animals. One had yielded positive blood and lymph node biopsy cultures during vaccination.

The fact that the transplant on passage through guinea pigs (like E.V. 51) acquired pathogenicity for mice deserves consideration. As so-called E.V. 76 Pf, it now has an LD_{50} of 3500 in intravenous inoculation, in contrast to the original subculture, which caused no deaths with 100,000 viable organisms. Since no more langurs are available, its virulence will be tested on African vervets when these become available. It is advised that the Commission on Immunization discuss and recommend further studies, particularly in human subjects, with the original E.V. 76 (Paris).

III. Studies on the pathogenicity and immunogenic efficacy of living P. pestis M23 in subhuman primates.

During the meeting of the Commission on Immunization and subsequently at the 69th Annual Meeting of the American Society for Microbiology, Mr. W. A. Janssen and Dr. M. J. Surgalla (Bacteriological Proceedings Abstracts M50, p. 75, 1969) reported on "Infectivity and Immunogenicity of a Noncapsulated Strain of Pasteurella pestis." The pigmented, noncapsulated (F_1-) variant selected from the isolate M23 described by Burrows (1957) was fully virulent for mice, but reduced in virulence for guinea pigs. When tested on rhesus monkeys (Macaca mulatta), it caused no serious illness even when administered in relatively large doses. Monkeys infected with the M23 variant became resistant to typical virulent strains in doses sufficient to cause lethal bubonic or pneumonic plague in non-immune monkeys. The results were impressive, but in view of the facts that the strain was fully pathogenic for mice and that protection tests had been executed on macaques, the principal investigator was requested to repeat the experiments using other subhuman primates. A stable freeze-dried preparation of a nonpigmented mutant M23, manufactured by the Walter Reed Institute for Medical Research and carefully selected in respect to certain biochemical and nutritional markers, became available in January 1969.

Following intraperitoneal or subcutaneous inoculation of guinea pigs, the PD_{50} of the freeze-dried preparation varied between 1300 and 2200. Compared with E.V. 51f, the immunogenicity of M23 for guinea pigs was slightly inferior. The same preparation as used on the guinea pigs in the dosage of 170 viable P. pestis proved lethal to 50% of mice inoculated intravenously and conferred inadequate protection.

By rearranging contemplated experiments, small groups of African green monkeys (Cercopithecus aethiops Kenya), langurs (Presbytis entellus) and macaques (Macaca mulatta) were assembled and inoculated by the subcutaneous or intradermal route with 38 million or 40 million viable M23 organisms each. Regrettably, titrations could not be performed. Observations in the course of these experiments are detailed in Table 9.

Differences in the susceptibility of the three subhuman primate species were again demonstrated. Strain M23 is infective and invasive, but even in the dose of 38 million viable P. pestis, local and systemic reactions in the Macaca were slight, although enlargement of the regional lymph nodes following intracutaneous vaccination was more marked than with E.V. 51f. None of the vaccinated Macaca were clinically ill, rises in temperature were slight, and local reactions completely disappeared between the 10th and 14th day. Reactions in the Kenya Cercopithecus were quite different: Two of four inoculated by the intradermal route succumbed to vaccination. All, including those vaccinated by the subcutaneous route, were clinically ill, lost weight, and had diarrhea; some displayed slight rises in body temperature and yielded positive

TABLE 9 - PART 1

Clinical and Pathological Effect of Freeze-Dried Living M23 (Non-Pigmented W.R.) in Subhuman Primates

Species Number and Dosage		Systemic Reactions	
		Temperature	Blood Culture (day)
38 million			
CA 234	sbc.	38.3 - 39.6	+5. L: 25,000
CA 233	sbc.	38.3 - 39.6	+5, +8. L: 33,600
CA 248	I.	38.3 - 39.8	+5, +8. L: 34,000
CA 237	I.	38.4 - 39.4	+2, +5, +8. L: 26,000
CA 247	I.	38.8 - 40.1	++5, +8. L: 18,000
CA 238	I.	38.4 - 39.7	+5, +8. L: 28,000
40 million			
SE 316	I.	39.2 - 40 (5)	02, +++5, +++7. L: 18,000
SE 300	I.	38.3 - 40.1	02, +++5, ++7. L: 36,000
SE 333	I.	39.2 - 40.2	02, +7. L: 23,000
SE 334	I.	39.1 - 40.2	02, +7. L: 23,000
38 million			
MA 5305	sbc.	37.9 - 38.5	0. L: 20,000
MA 5447	sbc.	38.5 - 38.8	0. L: 24,000
MA 5918	I.	38.4 - 39.8	0 L: 21,000
MA 5443	I.	39.0 - 39.9	0 L: 21,000
MA 5286	I.	38.9 - 39.7	0 L: 29,000
MA 5299	I.	39.1 - 39.7	0. L: 20,000

CA = *Cercopithecus aethiops*, Kenya
SE = langur, *Presbytis entellus*
MA = *Macaca mulatta*
sbc. - subcutaneously, xiphoid
I = intradermally, femur

02 = negative, 2nd day
+5 = 2-10 colonies, 0.25 ml, 5th day
++ = 20 colonies, __ day
+++ = 50 colonies, __ day
L = leucocytosis

TABLE 9 - PART 2

Species Number	Local Reactions	
	Site of Inoculation	Lymph Nodes
CA 234	nodule, erythema, 3 x 3 cm	R & L axillary, 3 x 4 cm, hard
CA 233	nodule, erythema, 1.6 x 2 cm	R & L axillary, 4 x 4 cm & 2 x 2
CA 248	indurated nodule, 1.4 cm	L inguinal, 3 x 1 cm, hard
CA 237	.9 nodule, eschar	L inguinal, 2.9 x 1.7; R, 0
CA 247	nodule 1.4 x 1.4, eschar	L inguinal, 2.5 x 1.8; R, 0
CA 238	nodule 1.7 x 1.7, eschar	L inguinal, 2.8 x 1.5; R, 0
SE 316	indurated raised purplish nodule, 2.7 x 2.2	L inguinal, 4.0 x 2.5, hard, *P. pestis* +++5
SE 300	indurated nodule, 1.8 x 1.8	L inguinal double, 4 x 2.8, +++5 *P. pestis*
SE 333	nodule, 2 x 1.5, purplish	L inguinal, 4 x 3; R, 2 x 2, +++5 *P. pestis*
SE 334	nodule, 1.8 x 1.0, purple	L inguinal, 3.6 x 2.1; R, 2 x 2, +++5 *P. pestis*
MA 5305	nodule, 2 x 2 raised	no enlarged lymph node
MA 5447	nodule, 1.5 x 1.5	"
MA 5918	nodule, .8 x 0.8, purplish	"
MA 5443	nodule, .8 x 0.8, pale	L inguinal, 2.5 x 1.5
MA 5286	nodule, 1.2 x 1.2, eschar	L inguinal, 2.5 x 2.5
MA 5299	nodule, 0.9, eschar	L inguinal, 2 x 2

TABLE 9 - PART 3

Species Number	Autopsy Day of Death and Anatomical Lesions	 Isolation of *P. pestis* M23
CA 234	--	--
CA 233	--	L axillary, 5 day ++
CA 248	--	L inguinal, 5 day ++
CA 237	7th, splenic tumor, chronic pulmonary abscess; lymphadenopathy	Heartblood (50), spleen, lymph nodes; lung - pseudomonas
CA 247	--	--
CA 238	17th, splenic tumor; lymphadenopathy, lung edema, enteritis	Lymph nodes, heartblood, spleen. Shigella intestines, *P. plasmodium gonderi*
SE 316	7th, splenic tumor; lymphadenopathy, liver necroses, enteritis	Heartblood +; lymph nodes, spleen; local
SE 300	7th, splenic tumor; lymphadenopathy, liver necroses	Heartblood ++; lymph nodes, spleen; local
SE 333	8th, splenic tumor; lymphadenopathy, few liver necroses	Heartblood +++; lymph nodes, spleen; local
SE 334	17th, local abscess and L inguinal lymph node; splenic tumor, hepatitis and nephritis, clotted blood in gallbladder	L inguinal & iliac lymph nodes, spleen, gallbladder content, heartblood
MA 5305		
MA 5447		
MA 5918		
MA 5443		
MA 5286		Biopsy lymph node, +++5, smear negative.
MA 5299		

blood cultures up to the 8th day (the last day tested, since repeated handling further impaired their health). Local lesions in the form of persistent nodules and prominently enlarged lymph nodes far exceeded those observed in animals vaccinated with E.V. 51f. The deaths resembled mortalities seen in virulent plague infections, except that the lymph nodes were never surrounded by edema or hemorrhages. In CA 237, an encapsulated chronic pulmonary abscess due to a Pseudomonas species may have weakened the Cercopithecus, but P. pestis (M23) was bacteriologically isolated from the heart-blood and viscera. The second fatality occurred in an animal which became ill on the 5th day, had diarrhea on the 8th day (latent Shigella infection and previously established Plasmodium gonderi), and, despite careful nursing, died on the 17th day with the lesions of lung edema and parenchymatous changes in the liver, kidneys and adrenals. As expected, P. pestis M23 was no longer demonstrable in the heart-blood, but it was present in the spleen and lymph nodes. The survivors' recovery from vaccination was very slow. The degree of protection will be tested after the expiration of five months.

The outcome of the vaccination of four langurs in excellent health when inoculated with M23 by the intradermal route was most disconcerting. By the 5th day, they were seriously ill; consequently daily examinations were discontinued. They all had fever and yielded blood cultures. Local dermal lesions were pronounced -- purplish, protruding, hard nodules, which in one animal (SE 334) revealed an abscess core by the 19th day. The regional lymph nodes were larger and more indurated than any previously seen in Cercopithecus or langurs vaccinated with E.V. 51f. Anorexia and extreme weakness combined with wasting and diarrhea preceded the animals' early deaths on the 11th (2) and 12th days. Despite special nursing, one animal (SE 334) also failed to recover. Autopsy findings were more severe than those observed in langurs vaccinated with E.V. 51f. Splenic tumor, adrenal hemorrhage and necrosis, and particularly diffuse liver necrosis and granulomas of varying extent, were pronounced. Enlargement of the inguinal and iliac lymph nodes without edema or hemorrhages but with liquefaction of the center cores was marked, surpassing that induced by E.V. 51f in this primate species. The gallbladders were greatly enlarged and filled with thick, tarry, blood-tinged material, barely resembling bile. Whether this overextension and concentration resulted from malfunction of the inflamed intestinal tract or from extensive pathology of the liver parenchyma has not been determined, since sections are not yet available. The gross anatomical lesions and marked leucocytosis with a shift to 18-22% monocytosis imply that rampant multiplication of the vaccine strain created toxic injuries not previously noted with E.V. 51f. Although nothing is known regarding the nature of the toxins formed by M23, by analogy, endotoxins are suspected. The absence of peritoneal and pleural transudates in lethal M23 vaccinations, in contrast to E.V. 51f infections, suggests some profound changes in water metabolism as a result of the nonspecific diarrhea and the inability or unwillingness of the animals to replace the loss by drinking water.

In one langur (SE 316), the intraperitoneal administration of glucose solution failed to prolong life. These observations must ultimately be extended by physiologic studies, preferably conducted in India, where langurs are readily available. However, the impression that M23 possesses not only "residual virulence" for langurs but also intriguing lethal properties not detectable when administered to guinea pigs, cannot be dismissed.

Antibody response thus far is disappointing. As expected, none of the *in vitro* tests for Fraction 1 yielded reactions. No mouse protective antibodies were found by *in vivo* tests, at least in guinea pigs, which, as a result of the vaccination, resisted infection. The C-reactive-protein tests, though confirmed, merely indicated that M23 induces fever and inflammatory reactions, attesting to the invasiveness of the attenuated *P. pestis*. In comparative tests conducted by W. A. Janssen, the sera of some of the vaccinated subhuman primates yielded positive reactions with the agar-gel precipitation inhibition test. Until the antigens responsible for the precipitins are identified, the significance of these antibodies in an indirect *in vitro* test reflecting the degree of immunity induced by M23 will remain undefined.

Preliminary vaccination studies with the freeze-dried, nonpigmented Fraction 1-deficient M23 in three subhuman primates disclosed that this vaccine is not only pathogenic but actually lethal for langurs and individual African greens but not for rhesus monkeys in the dosage of 28 million organisms, which is well tolerated by guinea pigs. Judging by the local and systemic reactions, which were more severe than those induced by E.V. 51f in langurs, it is logical to conclude that in man such reactions would probably be equally severe and not merely categorized as "unpleasant side effects", generally considered unimportant. Even if future experiments demonstrate that small-dose cutaneous vaccinations reduce the local cutaneous reaction, it remains to be proved by careful clinical studies that the pronounced invasiveness followed by inflammatory, necrotizing lesions in lymph nodes and particularly in the liver may also gravely impair the physiology of langurs. Measuring the levels of the C-reactive protein test in the sera of surviving macaques and vervets may be informative. Immunologists who recall the dictum "Nihil nocere" from the Hippocratic oath may well ponder whether the use of live plague vaccines, proven to induce a highly efficient cellular immunity in experiments with guinea pigs, is really warranted or justified. The question raised by Burrows (1957): "It is conjectural whether, in man, M23 would behave as it does in the mouse or as in the guinea pig." has been answered indirectly by the mortality rates in langurs and vervets. Until the pathophysiologic injuries produced by M23 are better understood, further experiments with the ultimate goal of using this isolate for the protection of man against plague have been discontinued.

On the assumption that further experimentation will disclose that solid protection against aerosol pulmonary plague infections can only be secured with live vaccines, explorations employing some definite form and schedule of vaccination with killed vaccines followed by live vaccine inoculations should be undertaken. Recent practices in smallpox vaccination have clarified the character and spacing schedules of vaccines which proved harmless and highly efficacious. Control of vaccination side-effects with the use of antimicrobial drugs seems appropriate, but from experience acquired in the chemotherapy of human plague, it is doubtful that injuries due to endotoxin can be prevented.

IV. Immunogenicity of Fraction 1 P. pestis antigen in langurs.

Protection experiments with the highly purified "envelope" antigen of P. pestis (called Fraction 1), originally isolated by Baker and his associates in 1947, proved that mice and rats, and, to a lesser extent, guinea pigs and macaques, could be protected against infection induced by wild plague strains. Clinical trials (Annual Report No. 3, 1953, p. 32-33) demonstrated that subcutaneous inoculations of the highly soluble antigen in a dosage of 1-3 mg stimulated mouse protective antibodies in significant concentrations in 50-62% of human subjects. In studies to protect rhesus monkeys against pneumonic plague, 5.5 mg of Fraction 1 given in repeated inoculations over a 170-day period (long-term moderate dose) resulted in a 4/4 resistance to severe challenge with no signs of disease in any of the animals (Ehrenkranz and Meyer, 1955, J. Infect. Dis. 96:138-144). Prior to experiments exploring the merits of combined vaccinations against plague with killed vaccines followed by live vaccines, Fraction 1 antigen, which in adjuvant #65 had a PD_{50} of 55 micrograms in guinea pigs, was tested for efficacy on langurs. Pilot tests on green vervets are still in progress.

A group of 10 langurs was inoculated subcutaneously with single 1 mg doses of Fraction 1 (B. 1968-I). Hemagglutinin titers determined on the 61st day after inoculation were relatively low except in two individuals (see Table 10).

Six animals of the 10-member group were each re-inoculated with 1 or 2 mg of different Fraction (B. 1968-I) preparations (kindly supplied by Dr. E. E. Baker), either subcutaneously or intramuscularly, at monthly intervals, and finally in the left femoral muscle with an inoculation of 1 mg emulsified in adjuvant 65 (kindly supplied by Dr. M. R. Hilleman, Division of Virus and Cell Biology Research, Merck Institute for Therapeutic Research). Local reactions following the adjuvant vaccine were negligible, but in several individuals, the regional inguinal lymph nodes enlarged -- a factor which persisted for more than 30 days. Little or no changes in HA titers were recorded on the 28th day after the injection or adjuvant-treated antigen. MPI readings are not yet available. Infection produced subcutaneously with 342,000 P. pestis 195/P indicated that the protection conferred by Fraction 1 was of a low order. Of the three langurs each inoculated with a single dose of 1 mg of the antigen, one died on the same day as the controls on the 4th day after challenge with the lesions of acute plague. Langur SE 336 exhibited a local lesion, became ill, and finally died on the 18th day in an emaciated state, with a skin ulcer, axillary abscess and metastatic lobar pneumonia of the right anterior lobe. At the time of this report, the third animal of this group reveals draining abscesses at the site of infection and the right axillary lymph nodes. The fourth langur, which demonstrated no significant antibody levels, became lethally infected when inoculated with the F_1 negative attenuated P. pestis M23 isolate.

TABLE 10

Immunogenicity of Fraction 1 in Langurs

Animal Number	Inoculation Schedule	Antibody Titers Days Post Basic Immunization						Response to Infection with 343,000 P. pestis
		61	91	135	178			
		HA	HA	HA	HA	CF	MPI	
SE 297	1, 2, 3, 4	256	4096	256	256	2		S. Local abscess, no lymphadenopathy
SE 320	1, 2, 3, 4	4	16	16	64	0		D 14. Bubonic plague, granulomas, arthritis
SE 324	1, 2, 3, 4	64	128	128	512	0		D 10. Bubonic plague, secondary pneumonia
SE 327	1, 2, 3, 4	32	128	512	64	0		S. No local reaction
SE 328	1, 2, 3, 4	4	0	32	512	0		D 10. Bubonic plague, secondary pneumonia
SE 329	1, 2, 3, 4	16	0	0	0	0		D 10. Bubonic plague, secondary pneumonia
SE 298	1	4	-	-	0	0		D 4. Acute bubonic plague.
SE 303	1	16	-	-	0	0		S. Local and axillary abscess, 25th day.
SE 336	1	256	-	-	0	0		D 18. Purulent axillary lymphadenitis, pneumonia.

Inoculation schedule:

1. Fract. B. 1968 I, Sbc.
2. Fract. B. 1969 I, Sbc.
3. Fract. B. 1969 I, I.M.
4. Fract. B. 1969 I, + adjuvant 65, I.M., PD_{50} - 55 micrograms guinea pig

S = Survived
D = Day of Death

Four langurs each inoculated over a period of five months with a total of 5 mg of Fraction 1 responded to infection by dying belatedly on the 12th (3) and 14th days: these mortalities occurred 8 to 10 days after the deaths of the acutely ill controls. At autopsy, all animals presented skin lesions, suppurating axillary lymph nodes, splenic tumors, metastatic, necrotic pulmonary infarcts surrounded by pneumonic consolidation of varying degree, and, in one, purulent arthritis of the left knee-joint, with isolation of P. pestis from the grossly diseased tissues including the heart-blood. Two langurs (SE 297 and 327), with CF antibodies of low titer after the 3rd inoculation of antigen, remained well without local reactions at the site of infection, as was the case with animals vaccinated with the live E.V. 76 (Paris) vaccine, included in the challenge series in order to economize on controls. Protection against death conferred upon langurs with either the E.V. 76 (Paris) or E.V. 51 was complete (100%), while with the antigen Fraction 1, it was only 33%. Correlations between MPI and protective efficacy may answer two questions: Is the lack of immunity (or unsufficient immunity) in some way connected with (a) "immuno-paralysis" as observed in guinea pigs, or (b) with inherent inadequacies of the chemical plague antigen vaccine? When the results of similar series of inoculation experiments with Fraction 1 in African green monkeys are available, the answers may be forthcoming in the quarterly reports. The conviction that Fraction 1 is the main protective antigen in mice and guinea pigs against infection provoked by wild strains of P. pestis has been weakened by results of experiments with langurs, and thus requires re-evaluation.

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13. ABSTRACT

In pilot experiments involving 15 langurs vaccinated with 420 million and 42 million freeze-dried viable P. pestis E.V. (WR-S) absolute protection persisted in 10/15 individual animals for at least 16 months. Living P. pestis vaccine E.V. 51f perfectly safe for guinea pigs and mice caused vaccination fatalities in vervets Cercopithecus aethiops Ethiopia, 3/16 or 18%; C. aethiops Kenya, 10/20 or 50%, C. aethiops pygerthrus, 13/50 or 26%; and langurs (Presbytis entellus) 2/4 or 50%. Surviving Ethiopia and Kenya vervets and langurs were solidly protected but 3/10 C. pygerthrus lost immunity by the 5th month. Fraction 1-deficient, nonpigmented living P. pestis M23 was lethal to 4/4 langurs in the dosage of 40 million well tolerated by guinea pigs. It was also pathogenic for Cercopithecus, but not rhesus monkeys. It is a "harmful" living vaccine and protects only 3/6 Cercopithecus. Fraction 1 in adjuvant in 5 mg doses protected only 2/6 langurs. Freeze-dried attenuated living P. pestis E.V. (WR-S) vaccine conferred protection to 10/15 langurs for 16 months. Formalin-killed freeze-dried E.V. 51f protects mice and guinea pigs as U.S.P. vaccine. Freeze-dried attenuated plague isolate E.V. 76 Paris originally isolated by G. Girard is highly protective for mice, guinea pigs, langurs, vervets and macaques; it is a "safe" vaccine since it produced no deaths in monkeys, but local and systemic reaction with dosages of 38-40 million viable organisms. It may prove to be the only relatively harmless living vaccine. Antibody response in langurs is modest.

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MECHANISMS OF HYPERSENSITIVITY PHENOMENA

ANNUAL PROGRESS REPORT

by

Abraham G. Osler, Ph.D.

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SUMMARY

Studies of the allergic response of rabbit platelets have led to the findings that leukocytes from immunized rabbits carry reagin-like antibodies. These cells release histamine on interaction with specific antigen. Although lymphocytes from nonimmunized rabbits contain no histamine, the reaction of these cells with antigen leads to histamine release and membrane disruption of platelets in the reaction mixtures.

Guinea pig gamma-1 and gamma-2 immunoglobulins appear to utilize different pathways in their attack on the complement system and as mediators of complement dependent allergic reactions of the immediate type.

Methods are being developed for antibody weight estimates at the nanogram level, based on the union of antibody with specific hapten.

A. Allergic Histamine Release from Rabbit Cells

1. Rabbit Leukocytes

In the course of studies on the allergic response of rabbit platelets, the results of several experiments suggested that rabbit leukocytes might participate in a reaginic type of histamine release. These observations have been extended with the following results. Suspensions of washed leukocytes obtained from immunized rabbits release histamine on admixture with antigen, in the absence of added serum. Reactive cells are obtained within the first week after a single injection of antigen, and persist for two to three months. The release process is mediated by calcium without apparent membrane damage. In several other respects the findings obtained with the rabbit leukocytes are entirely comparable to the IgE-bearing human white cells. It is of interest that reactive leukocytes have been obtained from all rabbits following parenteral immunization with a variety of antigens. The *in vitro* release of histamine from rabbit cells is more sensitive than the passive transfer reaction in rabbits (PCA), (Table I). Blood lymphocytes do not participate in this response since these cells lack histamine. Lymphocytes are actively involved however in another type of immediate allergic reactions as described below.

Lymphocyte-platelet interaction

One of the experimental difficulties in studies of allergic reactions with rabbit cells stems from the relatively high concentration of histamine in the platelets. In the leukocyte studies referred to above, it was therefore essential to establish that the leukocytes were indeed the source of the antigenically induced histamine release. This fact was soon established by a series of experiments which also uncovered the following, rather novel phenomenon. Platelets from non-immunized rabbits are destroyed in reaction mixtures containing sensitized leukocytes and antigen. Schoenbechler and Barbaro also studied this phenomenon and concluded that lymphocytes were involved, a finding we have confirmed. It is therefore apparent that sensitized lymphocytes, which do not contain histamine, react with antigen and destroy neighboring platelets. We have previously documented the observation that platelets furnished by immunized rabbits do not carry immunoglobulins capable of mediating histamine release. It follows therefore that the lymphocyte-platelet interaction provides an interesting example of a phenomenon which is cytotoxic for platelets and which is mediated indirectly by an immunoglobulin on sensitized lymphocytes. The nature of this immunoglobulin and the mechanism of this reaction is under investigation.

B. Biologic Activities of Guinea Pig Immunoglobulins.

Gamma-1 and Gamma-2 immunoglobulin rich fractions, isolated from guinea pig antisera to proteins and to dinitrophenylated proteins, were compared for their biologic properties. As previously reported by Benacerraf and coworkers, the γ-1 immunoglobulins mediate PCA in the guinea pig skin and fail to activate the complement (C) system in conventional type assays. However, these antibodies do fix guinea pig (and rat) C when used as washed preformed aggregates. As suggested by the data in Table II, the attack on C by γ-1 complexes seems to differ from the well known sequence in which antibody and antigen interact successively with the C

components in the order; C1, 4, 2, 3, 5, 6, 7, 8 and 9. The γ-1 aggregates apparently bypass the early components (C1, 4, 2) and enter the sequence at the C3 stage. It is of course difficult to exclude the possibility that minimal activation ($\leq$ 2%) of C1 does indeed occur, but our data also support alternative interpretations. One hypothesis now under study is that the aggregates of guinea pig γ-1 immunoglobulins activate a serum enzyme which is not one of the recognized C components but which can utilize C3 as its substrate. The possibility is also entertained that all immune complexes may utilize this pathway, in addition to the classical sequence noted above, while the guinea pig γ-1 antibodies are limited to the bypass mechanism. The existence of an alternate pathway for C activation is not without precedent but its precise nature awaits characterization. The preferential interaction of C3 with yeast cell wall and endotoxin preparations may be pertinent. Studies with guinea pig γ-1 immunoglobulins indicate that the tissue damaging consequences of in vivo immune reactions may also proceed by more than one route. Thus, the attack on C3 by specific immune aggregates containing guinea pig γ-1 immunoglobulins is associated with anaphylatoxin production, histamine release from rabbit platelets and the induction of Arthus reactions. In all of these instances, the γ-2 immunoglobulins, isolated from the same serum pool, are more potent tissue-damaging agents, on a weight basis, as shown in Tables III-V.

Antibody Weight Assays at the Nanogram Level

In 1966 we described a modified Farr procedure for weight estimates of antibody at the microgram level. The utility of the method arose from the fact that all immunoglobulin molecules capable of binding with antigen were registered, despite their inability to agglutinate tanned red cells, fix complement or mediate cutaneous anaphylaxis. Application of the assay to early antibody production in vivo showed a simultaneous synthesis of 7S and 19S immunoglobulins, the former, frequently in great molar excess. These reports, and those from other laboratories brought into serious question the widely accepted thesis regarding the primacy of 19S antibody production.

We have since attempted to increase the definition of the method with respect to the sensitivity and specificity. To attain the former, we hoped to achieve successful measurements at the nanogram level. For greater specificity, we limit the immune event to a small number of antigenic determinants, possibly, one. The immune system under study involves the interaction of guinea pig and rabbit antibodies to dinitrophenylated proteins with the hapten, dinitrolysyltyrosine (DNP-LT).

The principle of the procedure is similar to that employed in the earlier mABC analyses. Dilutions of antisera are mixed with varying concentrations of radio-iodinated DNP-LT, in large molar excess over the antibody input.

The rationale of the procedure is to drive the union of antibody with hapten to the region of marked hapten excess so as to yield the limit complex of H_2 Ab,

where H = DNP-LTI^{125}
and Ab = antibody.

The complexes are precipitated and washed with half-saturated ammonium sulfate, and their content of radio-iodinated DNP-LT is estimated. If the immune complexes have attained the limiting configuration of H_2 Ab, then the amount of iso-

topically labeled hapten precipitated by half-saturated ammonium sulfate should be independent of the hapten input. Under these conditions, the molarity of precipitated hapten is twice that of the antibody input. Since the specific activity of the iodinated hapten, DNP-LTI^{125}, equals or exceeds 10^{-6} cpm per 10^{-10} M, antibody estimates at the nanogram level become attainable. Although we have had a number of successful experiments, the assay is still plagued by occasional and sporadic nonspecific binding, whose cause has not been identified.

Estimates of antibody gained by means of the hapten binding assay generally exceed the precipitin values by 50 to 100 percent. At this writing, we do not know whether the higher values can be attributed to nonprecipitating antibodies, as was the case for the earlier, m/ABC assay, or to non-immune binding of radioiodinated compounds. The sensitivity of the method may be illustrated by the statement that an immune serum containing 100 μg of precipitable anti-DNP N per ml, may be diluted 10^5-fold and still yield a relatively valid assay of antibody content without prior isolation of the immunoglobulins.

The potential utility of the procedure is exemplified by its sensitivity (≤ 0.001 μg of antibody N), by its lack of dependence on the secondary manifestations of antigen-antibody interaction (e.g., aggregation, virus neutralization, complement fixation, etc.), and by the ability to study antibody synthesis to one or a minimal number of determinants.

Additional experience with the procedure should permit meaningful studies of cellular immunology.

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TABLE I

The Release of Histamine from Suspensions of Washed Rabbit Leukocytes

Days after injection	No. of rabbits	No. of sera yielding homologous PCA reactions	No. of rabbits whose WBC released histamine with antigen	Mean histamine release	Maximal histamine release for each leukocyte suspension
				% (S.E.)	
3	4	0	0	0	0,0,0,0
5	4	0	2	17 (1.5)	15, 18
7	4	1	1	16	16
8	3	2	2	34 (17.5)	15, 51
9	11	4	10	58 (10.6)	90,55,56,96,94, 86,23,53,11,13
10	8	3	8	51 (10.6)	52,60,18,100, 63,45,20,20
11	3	1	3	50 (20.8)	85,53,13
14	4	1	2	59 (29)	84, 26
18	3	1	2	60 (14.5)	74, 45
21	4	3	4	62 (11.3)	91,63,36,57
25-28	7	4	5	52 (5.9)	44,63,33,55,64
36-43	7	4	4	45 (8.3)	44,28,68,41
52	4	1	3	28 (9.8)	19,18,48

Rabbits were immunized on day 0 with 10 mg of either HSA or Ea in complete Freund's adjuvant.

Isolated washed leukocytes from these animals were tested for histamine release with 3 different levels of the immunizing antigen, i.e., 1.0, 0.1 and 0.01 μg N/ml. Specificity of the reaction was tested by addition of HSA to the cells of rabbits immunized with Ea, and vice versa.

Sera from the bleedings taken for WBC isolation were also used for PCA tests, either undiluted or at a 1 → 5 dilution.
Positive histamine release $\geq$ 10%.

TABLE II

Complement Fixation by Immunoglobulins
in Hyperimmune Guinea Pig Anti-DNPBGG

Order of addition: Antibody, C, Antigen				Preformed immune aggregates** + C		
Antibody		Ratio	CH_{50} fixed %			
Preparation	Amount µg N*	Ab N / DNP HSA N		Preparation	Amount µg N*	CH_{50} fixed %
Serum	6.7	10	43	Serum	32	>90
	3.5	11	24		7	56
					5	37
					2.5	14
A (γ-2a)	6.7	10	>90	A (γ-2a)	7	>90
	1.9	12	31		4	51
					2	32
					1	11
H (γ-1a)	8.2	12	7	H (γ-1a)	57	51
	6.6	20	0[a]		11	36
					5	14
					2.5	8
H_1 (γ-1b)	6.7	10	3	H_1 (γ-1b)	53	48
	6.3	19	0[a]		11	31
					5	14

* Anti-DNPBGG N per ml of undiluted guinea pig serum used as complement. (Either 1.5 or 2.0 ml C used).

** Formed at a ratio of guinea pig anti-DNPBGG N to DNPHSA N = 10.

[a] CH_{50} for control and fixation mixtures containing H and H_1 were; 177, 177, and 177 respectively.

TABLE III

Reverse Passive Arthus Reactions in the Guinea Pig Skin Induced by Homologous Immunoglobulins

Antibody preparation*	Antibody at skin site, μg N	Intensity of reaction after	
		4 hr	24 hr**
Untreated serum	45	++	
Pseudoglobulin	30	++	++++
A (γ-2a)	30	+++	+++
H (γ-1a)	65	++	+++
H_1 (γ-1b)	45	++	

* Hyperimmune guinea pig antisera to $DNP_{31}BGG$ and $DNP_{30}HSA$. The antibody was injected intradermally within minutes following intravenous inoculation of 1 mg $DNP_{30}HSA$ N.

** The 24 hr reactions were markedly more hemorrhagic than those observed at the 4 hr period.

TABLE IV

Histamine Release and ^{86}Rubidium Efflux from
Rabbit Platelets by Guinea Pig Immunoglobulins

Antibody preparation	Electrophoretic class	Histamine release	^{86}Rb efflux
		percent	
Guinea pig anti-DNPBGG (hyperimmune)			
Serum		51	52
Pseudoglobulin	γ-2 and γ-1	59	92
A	γ-a	84	100
H	γ-1a	61	100
H_1	γ-1b	88	100
Guinea pig anti-HSA			
Serum			
A	γ-2	65	62
H	γ-1a	51	50
H_1	γ-1b	11	14

Each reaction mixture contained:
Antibody - 20 μg N/ml   Antigen - 1 μg N/ml
Plasma - 10 percent   Added Mg^{2+} - 5×10^{-4}M
Plasma contribution - Mg^{2+} - 1×10^{-4}M, Ca^{2+} 3.8×10^{-4}M

TABLE V

Anaphylatoxin Production in Guinea Pig Serum by Homologous Immunoglobulins

Anti-DNPBGG (Early)			Anti-HSA (Hyperimmune)		
Antibody preparation	Amount*	Diameter of blueing mm	Antibody preparation	Amount*	Diameter of blueing mm
Pseudoglobulin	52	17	Pseudoglobulin	50	14
A (γ-2)	49	14	A (γ-2)	51	13
H (γ-1)	50	8	H (γ-1)	51	10
None	--	5	None	--	5

* μg antibody N in 2.0 ml of guinea pig serum.

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Hypersensitivity, allergy, platelets, leukocytes, histamine, complement, antibodies, antigen binding						

AD ____________________

ANTIBODY FORMATION (U)

ANNUAL PROGRESS REPORT

by

Jonathan W. Uhr, M.D.

August 1969
(For the period 1 July 1968 to 31 May 1969)

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Office of The Surgeon General, Washington,D.C. 20315
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SUMMARY

Title of Project: Antibody Formation

Principal Investigator: Jonathan W. Uhr, M.D.

Preparing Institution: New York University School of Medicine

Contract number: DA-49-193-MD 2043

Supported by: U.S. Army Medical Research and
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Annual Progress Report to the Commission on Immunization of the Armed Forces Epidemiological Board, for the period 1 July 1968 to 31 May 1969.

Synthesis, Transport and Secretion of Immunoglobulin. Using plasma cell tumors in mice, incorporation of the carbohydrate portion of immunoglobulin and its intracellular transport has been studied. Both biochemical and ultrastructure radioautography techniques were used. The results indicate that some residues of glucosamine (the "bridge" sugar) are incorporated on polyribosomes in the rough endoplasmic reticulum whereas other glucosamine residues and galactose are incorporated in the Golgi complex. Immunoglobulin is transported from rough endoplasmic reticulum to Golgi complex, back over the cytoplasm of the cell and across the plasma membrane, perhaps by reverse pinocytosis.

Regulation of Antibody Synthesis by Serum Antibody. This mechanism has been studied by a "subtraction" experiment for the first time, i.e., by removal of specific antibody. This was accomplished in rabbits immunized to two antigens in saline 2-3 weeks previously by exposing their removed plasma to an immunoadsorbent containing one of the antigens before returning the adsorbed plasma to the rabbit. By this technique 50-75^{o}/o of antibody activity was removed specifically. Twenty-four to forty-eight hours later, serum antibody level to this antigen in question began to rise and reached a peak 5 days later. Serum antibody levels to the "other" antigen did not change. No evidence was obtained that significant amounts of antigen were released from the immunoadsorbent. The results suggest that serum antibody regulates the antibody response to metabolizable antigens.

Streptococcal Infections in Chimpanzees. Streptococcal infections have been induced in 12 chimpanzees by injections of group A strains into the pharynx and skin. All animals developed a brisk ASO response. No definite evidence of sequelae has been obtained as yet. Studies are now in progress in which chimpanzees previously immunized to M protein preparations are being infected with streptococci.

Synthesis, Transport and Secretion of Immunoglobulin

During the last year, the intracellular sites of addition of the carbohydrate moieties have been elucidated as well as part of the intracellular route of transport of the immunoglobulin molecule. Three approaches were used in this study, all based on pulse-labeling of plasma cells from a mouse myeloma tumor with ^{3}H-glucosamine (a "bridge" sugar), galactose or leucine. The first approach was to determine the extent to which puromycin prevented incorporation into immunoglobulin. The rationale was as follows: If the given sugar is incorporated on nascent polypeptide chains only, puromycin should completely prevent such incorporation. If, on the other hand, the sugars are added on completed chains late in the intracellular life of the molecule, then puromycin should reduce incorporation only partially for a given short period of incubation, since chains completed prior to puromycin addition continue to have the sugars attached to them.

The results of the puromycin experiments are summarized in Table I.

As can be seen in Table I, puromycin prevented IgG synthesis. In contrast, incorporation of glucosamine and galactose into IgG was not completely abolished. Thus, a portion of the residues of each of these two sugars is incorporated into completed polypeptide chains. The effect of puromycin was more pronounced on glucosamine incorporation into intracellular IgG than on galactose incorporation. This is particularly apparent after 60 minutes of incubation when 89% of glucosamine incorporation into IgG has been prevented by puromycin compared to 63% for galactose. This observation is consistent with the possibility that some of the glucosamine is also being added onto nascent chains.

The second approach was to determine the percentage of total radioactive immunoglobulin that has been secreted after different periods of labeling with the above isotopes. This percentage should also give information on the time of incorporation of the sugars. Thus, if the incorporation of the sugars occurs on nascent chains, the proportion secreted should be similar to that of leucine labeled immunoglobulin, whereas if the sugar is added "late", a higher percentage of sugar labeled immunoglobulin should be secreted.

As can be seen in Table II, IgG labeled by either galactose or glucosamine appears more rapidly in secretions than that labeled by leucine. Thus, after 30 minutes of labeling, virtually no leucine-labeled IgG has been secreted ($4^{0}/_{0}$); in contrast, $30^{0}/_{0}$ and $50^{0}/_{0}$ respectively of glucosamine and galactose labeled IgG has been secreted. After 60 minutes of labeling, $15^{0}/_{0}$ of leucine-labeled IgG has been secreted whereas $75^{0}/_{0}$ of galactose-labeled IgG and $40^{0}/_{0}$ of glucosamine-labeled IgG are in the secretions. These experiments indicate that a portion of the galactose and glucosamine residues are added sometime after the IgG chains are synthesized, i.e., nearer to the time of their secretion. Again, there is a striking difference between glucosamine and galactose, i.e., a smaller proportion of glucosamine-labeled IgG is secreted compared to galactose-labeled IgG at both time periods studied. This observation is consistent with the interpretation of the puromycin studies which is that glucosamine is attached on both nascent and completed chains whereas galactose is added only on completed chains.

The validity of the measurements in the preceding labeling experiments depends upon the unambiguous demonstration that the radioactivity in IgG is covalently bound and is due to the particular sugar used rather than to some metabolic transformation product.

These questions were investigated by analyzing glucosamine- and galactose-labeled lysates and secretions by Sephadex, Dowex, and paper chromatography. The results indicate that incorporated radioactivity with the sugars represents covalent binding of glucosamine (in its acetyl form) and galactose respectively. Of particular interest was the unexpected finding that over $95^{0}/_{0}$ of incorporation of these two sugars into macromolecules in the cytoplasm during one hour incubation with the respective isotopes represented incorporation into immunoglobulin. These observations, therefore, provided a background for autoradiographic studies.

Thus, the next approach was to examine cells pulse-labeled as above by ultrastructure radioautography. This approach provided information on the intracellular site for the "late" addition of galactose and glucosamine, the intracellular route of transport of the immunoglobulin molecule, and the possibility that some glucosamine residues are incorporated on polyribosomes.

The results of these studies are summarized in Table III. After three minutes labeling with leucine, the majority of grains were seen over the rough endoplasmic reticulum of the cytoplasm in which the polyribosomes are present. There was relatively little labeling of the Golgi complex. However, after thirty minutes of cold amino acid chase, the labeling over the Golgi complex had increased almost ten-fold whereas that over the cytoplasm had been reduced approximately fifty percent. Thus, the route of transport of the immunoglobulin molecules is from rough endoplasmic reticulum to Golgi complex as was previously suggested by Clark in studies of rabbit lymph node cells (Clark, S.L., Jr., Am.J.Anat., 119, 375, 1966).

In contrast to leucine, galactose was incorporated primarily in the Golgi complex as early as five minutes after labeling and also at 15 and 60 minutes of labeling.

Glucosamine labeling was markedly different from that of galactose and looked like leucine, i.e., the grains were distributed over the cytoplasm as well the Golgi.

The above observations are entirely consistent therefore with the preceding biosynthetic studies. Taken together the results suggest a concept of IgG synthesis and secretion by LPC_1 plasma cells: (i) Synthesis of heavy and light chains begin on rough endoplasmic reticulum. (ii) "Bridge" glucosamine is added onto nascent polypeptide chains on rough endoplasmic reticulum. (iii) IgG is transported in the cisternae to the Golgi apparatus wherein galactose, additional glucosamine, and probably the remaining sugars are added onto the IgG molecule. (iv) The completed molecule is exported across the cytoplasmic matrix possibly contained in a microvesicle. (v) If the molecule is membrane-bounded, then transport across the plasma membrane is by reverse pinocytosis.

Regulation of Antibody Synthesis by Serum Antibody

This question was approached by removing antibody specifically from immunized animals. Rabbits were injected intravenously with bovine serum albumin (BSA) and bacteriophage T_2 (T_2). Two to three weeks later anti-BSA was removed from such animals by a procedure which involved exposure of removed plasma to an immunoadsorbent (^{125}I-BSA bound to bromoacetyl cellulose) and return of the adsorbed plasma to the animal. This resulted in removal of the majority of antibody activity to BSA without affecting antibody levels to T_2 (Fig. 1). One to two days later, anti-BSA levels began to rise and reached peak levels usually 5 days after the removal of antibody. Antibody levels to T_2 did not change. No evidence was obtained that BSA was released from the immunoadsorbent into the circulation of the rabbits. Thus, only trace amounts of radioactivity were released into the plasma; a majority of the radioactivity was equally co-precipitable with BSA or human gamma globulin and their specific antibodies; the released material did not elute with BSA on gel filtration. The results are interpreted as evidence that serum antibody acts as a regulatory mechanism for antibody formation during the conventional antibody response to a metabolizable antigen.

Streptococcal Infections in Chimpanzees

A serologic survey of 76 chimpanzees in two primate colonies has revealed ASO titers of 200 or greater in 14 (18.4$^{0}/_{0}$). Such titers in humans are generally considered evidence of recent group A streptococcal infection. Beta-hemolytic streptococci of group A (M non-typeable, T-type 12 and 28) and of "human" group C were recovered from some of these chimpanzees' throats. High titers of antibodies against the group A polysaccharide corroborated the evidence for the occurrence of spontaneous group A streptococcal infections in captive chimpanzees.

A series of experimental streptococcal infections were given to 12 chimpanzees in a closed colony. In the first phase three strains of group A type 12 beta-hemolytic streptococcus were injected into the tonsils and instilled into the nares on day 0, 7, and 14. Urinalysis were done daily and all the animals were biopsied on or about day 30. All the animals developed a brisk ASO response, but no significant proteinuria, nor histologic evidence of glomerulonephritis.

In the second phase all the animals were given infections with multiple nephrotoxic strains, both in the throat and in the skin. In the third phase, still in progress, the chimpanzees were immunized with a partially purified type 3 M protein in incomplete Freund's adjuvant, and a month later were infected. This experimental protocol was suggested by the report of a high rheumatic fever attack rate after streptococcal infections in children immunized with an M protein vaccine prepared from this same type and strain (Massell, B.F. Circulation 38, Suppl. 6, 135, Oct. 1968). One of four animals thus treated developed a new apical systolic murmur which was considered organic by several observers. Heart catheterization and cineangiography, however, failed to evidence a regurgitant jet. Heart biopsy revealed normal myocardium. Urinalyses and kidney biopsies did not reveal evidence of glomerulonephritis.

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TABLE I

Effect of Puromycin on Incorporation of Leucine and Sugars into Immunoglobulin

Isotope-^{3}H	Effect of Puromycin* %/₀ decrease in CPM incorporated			
	30 min		60 min	
	Intracellular	Secretion	Intracellular	Secretion
Leucine	100	100	100	100
Galactose	55	50	63	58
Glucosamine	75	58	89	53

* 6 X 10^{-4} M, 5 minutes before labeling

TABLE II

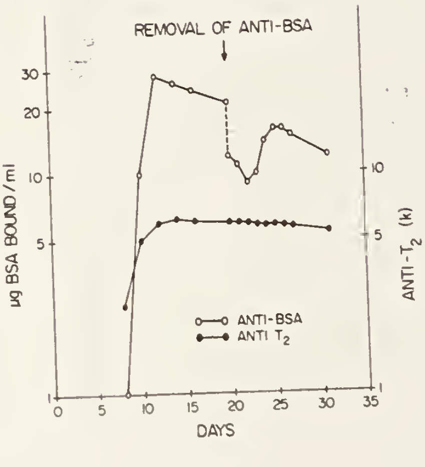
Percentage of Total Radioactive Immunoglobulin (IgG) Secreted after Labeling with Leucine and Sugars

Isotope-^3H	% Total radioactive IgG secreted	
	30 min	60 min
Leucine	4	15
Galactose	50	72
Glucosamine	32	40

TABLE III

	CELLULAR DISTRIBUTION %			
RADIOACTIVE PRECURSOR (^{3}H)	LEUCINE		GALACTOSE	GLUCOSAMINE
"PULSE" (MIN.) "CHASE" "	3 0	3 30	60 0	60 0
CYTOPLASM	57	28	16	41
GOLGI COMPLEX	3	27	51	12
NUMBER OF COUNTED GRAINS	1656	1326	483	872

Fig. 1


REMOVAL OF ANTI-BSA
µg BSA BOUND / ml
30
20
10
5
1
ANTI-T_2 (k)
10
5
1
0
5
10
15
20
25
30
35
DAYS
ANTI-BSA
ANTI T_2

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RESPONSES OF HUMANS TO CHOLERA VACCINE IMMUNIZATION (U)

ANNUAL PROGRESS REPORT

by

W. F. Verwey, Sc.D.

September, 1969

(For the period 1 August 1968 to 31 July 1969)

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The University of Texas Medical Branch
Galveston, Texas 77550

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SUMMARY

This study of the vibriocidal response to initial immunization and reimmunization with cholera vaccine employs four groups of volunteers, three of which have been immunized with two doses, 4 weeks apart, and one which received two weekly doses. The three groups will be reimmunized at intervals of 6 months, 1 and 2 years; and the group immunized at the 1-week interval will be reimmunized at 2 years.

No data are yet available concerning reimmunization. However, comparisons of response to initial immunization with two doses at 1-week or 4-week intervals have resulted in the following findings:

1. In confirmation of results obtained under a previous contract, the response to initial immunization following the second dose at 4 weeks is generally lower than the response to the first dose and no booster effect is seen.

2. Initial immunization consisting of two doses given at a 1-week interval, produces group mean titer responses that are at least equivalent to or (at most points) higher than the responses obtained with two doses given 4 weeks apart.

3. The comparison of group geometric mean titers 1, 2, and 6 weeks after the second dose demonstrates a very significant difference in favor of two doses given at weekly intervals.

4. The use of two doses 1 week apart is of greatest advantage in the immunization of persons who do not have pre-existing vibriocidal antibody. These persons show higher group mean titers and a decreased frequency of poor responders compared to those immunized with two doses 4 weeks apart.

FOREWORD

It is a pleasure to give grateful recognition to the cooperation and assistance of Dr. G. J. Beto, Dr. M. D. Hanson, Mr. S. A. Lanier, Mr. T. D. Hutto and Mr. W. D. Kutach of the Texas Department of Corrections. We wish to acknowledge especially the helpfulness of Capt. C. C. Aldridge in whose hospital unit all of the work with volunteers was done.

A variety of professional skills are required for the successful completion of research of this type. The participation of the following persons within The University of Texas Medical Branch is mentioned with real appreciation:

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Laboratory Immunology - Dr. Y. Watanabe
Dr. S. S. Rocha, Jr.
Mr. H. R. Williams, Jr.

Computation - Personnel of The University of Texas Medical Branch Research Computation Center, Dr. V. A. Benignus, Director

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council.

INTRODUCTION

In previous studies of the responses of human volunteers to cholera vaccine immunization carried on under Contract DADA 17-67-C-7084, groups of at least 17 human volunteers were used to study the effects of antigen quantity and the number and spacing of doses upon response to initial immunization; and the effects of dosage size and number on responses to reimmunization. The criterion of response to immunization was the tube dilution vibriocidal assay. Correlation between vibriocidal antibody levels and attack rates of naturally-acquired cholera have been reported by Mosley(1) for Inaba-type cholera in unimmunized populations, vaccine immunized populations and familial contacts of cholera cases. It would therefore appear that this test may be a relatively satisfactory indication of the state of immunity produced by bivalent vaccines.

In our previous work several observations were made which could have important implications in relation to cholera vaccine immunization practices and which appeared to require either confirmation or extension.

A. The currently recommended initial immunization procedure is the use of a 0.5 ml dose followed in approximately 4 weeks by a 1.0 ml dose. Our studies indicated that in most instances the response to the second dose was less than to the initial dose. A booster effect was definitely not observed.

B. The same two doses administered one week apart appeared to produce higher geometric mean titers and a decrease in frequency of "poor responders". Although this regime was numerically superior at all points in the immunization curve, the differences did not achieve statistical significance with the number of volunteers that were used in the initial study.

C. On reimmunization at 6 months, 0.5 ml of cholera vaccine produced maximum geometric mean vibriocidal titers that were significantly lower than those found on initial immunization. In addition there appeared to be an inverse correlation between the height of the initial response and the height of the response on reimmunization.

Since animal experimentation had demonstrated booster-type responses if antibody levels were allowed to fall to relatively low values, and since observations with a few individuals who had been immunized with cholera vaccine several years before had indicated high level responses, this work raised questions concerning the efficiency of every-6-month reimmunization.

The work undertaken under the present contract has been designed a) to investigate further the relative effectiveness of two doses of cholera vaccine 1 week apart, and two doses 4 weeks apart, using larger numbers of volunteers; and b) to investigate the response of individuals to reimmunization at 6-month intervals compared to responses to reimmunization at 1 year and 2 year intervals.

MATERIALS AND METHODS

Volunteers. The human volunteers used in this study were inmates of the Ramsey Unit of the Texas Department of Corrections, and were males between the ages of 21 and 35 years. Preimmunization bleedings were obtained from each individual, and the various groups that were created in this study were balanced in terms of the heights and frequencies of preimmunization vibriocidal antibody titers. The groups were similar in respect to race, height, weight, age and the frequency of military service.

Vaccine. In this study the same commercial vaccine used in the previous studies was employed for initial immunization. This vaccine was designated "CV" and in mouse protection tests was found to have protective potency approximately equivalent to the Ogawa and Inaba NIH Reference Vaccines. This vaccine would therefore be considered to be a "minimal satisfactory vaccine". A second lot of vaccine was required for the reimmunization portion of this study and, by good fortune, mouse tests have indicated a potency very similar to the first lot.

Plan of Study. Table 1 is a Flow Chart demonstrating the subdivision of the initial pool of volunteers into groups and indicates the injections and bleedings that each is scheduled to receive. All bleedings through 5/24/69 have been completed on schedule and titrations of blood samples collected through 12/21/68 have been completed. Each group initially consisted of 41 individuals. Group 1 represents current immunization practice, i.e., administration of two doses 4 weeks apart, with reimmunization at 6-month intervals. Groups 2 and 3 represent similar initial immunization but will receive reimmunization after a 1-year or 2-year interval, respectively. Group 4 received initial immunization with two doses a week apart, and will be reimmunized at the end of 2 years. Since Groups 1, 2 and 3 are identical in respect to their treatment during the first 6 months of the study, data from these groups can be combined to provide a baseline of approximately 123 people receiving initial immunization by the currently recommended technique, to be compared with a group of 41 persons receiving initial immunization with two doses of the same vaccine given 1 week apart.

Losses from a study of this type are unavoidable, primarily due to transfer and unexpected parole. However, since the volunteers selected for this study were drawn from prison inmates having relatively long sentences or histories not conducive to parole, losses have been minimal, and it is anticipated that most of the volunteers will continue to be available for the remainder of the study.

Vibriocidal Antibody Titrations. Vibriocidal antibody titrations have been performed by a tube dilution method similar to that described by McIntyre and Feeley (2). Vibrio cholerae strains V86 and 041 have been the Inaba and Ogawa test strains that have been used. A reference serum tested in duplicate is included in each day's testing and, with the exception of the preimmunization bleeding titrations, 6 to 8 consecutive sera from each volunteer have been assayed on the same day and a proportional number of volunteers from each group were included in each day's testing. Doubling dilutions of serum were employed in the test, and the endpoint was considered to be the location in the test (either a tube or an interpolated value between tubes) where approximately 50% growth inhibition occurred. As in our previous studies, a standard series of serum dilutions was established beginning at 1:2.5; therefore all results could be expressed in terms of tube numbers, which then represented a progression based upon a dilution logarithm to the base of 2. Geometric means for the various groups can then be calculated using endpoint tube numbers in direct arithmetic computations. Although 1:2.5 was the dilution of the initial tube on all preimmunization bleedings, 1:20 (tube 4 of the standard series) represented the initial dilution of all postimmunization samples. No samples so far obtained have failed to show an endpoint at least at this level.

Statistical and Computational Procedures. Vibriocidal titers against both Ogawa and Inaba cells were recorded on punchcards, which served as the input for an IBM 1800 computer which performed most of the statistical procedures that have been used in the analysis of these data. All geometric mean vibriocidal titers calculated in this study were accompanied by the simultaneous computation of the standard deviation and standard error for the group. Differences between means were tested for significance by the application of Students t test and were considered to be significant if values for P were equal to or less than 0.05.

RESULTS

The primary purpose of these experiments is to investigate the effects of reimmunization at intervals of 6 months, 1 year and 2 years. The first year of this experiment is not yet completed, so data concerning reimmunization are not available. For purposes of insuring comparability, bleedings that have been collected at 6 months from Group 1 (reimmunization at 6-month intervals) have been frozen and stored and will be assayed simultaneously with bleedings collected following 1 year's reimmunization of this group and Group 2 (reimmunization at 1-year intervals).

In addition to the principal objective mentioned above, however, this study can provide certain information on initial immunization supplementing that already obtained under Contract DADA 17-67-C-7084. Group 4 of the present study consists of 41 persons who received initial immunization using two doses 1 week apart, to be compared with the 123 persons in Groups 1, 2 and 3 receiving two doses 4 weeks apart. The initial immunization regimens of these groups are identical to those given to groups of 20 and 38 persons respectively that were studied in previous experiments. Figure 1 shows the geometric mean titer curves of the two groups through the first 10 weeks of the present experiment. Over this time period, overall results are in favor of the group receiving its second dose after 1 week. These curves are not only similar in shape to those obtained previously, but are practically identical in respect to their numerical values; therefore, point-by-point analyses were done comparing the corresponding values of the present and previous experiments. Since none showed significant deviations in either geometric mean titers or in the variance of these titers, it was concluded that the data obtained from the earlier experiment could be pooled with the present data to permit the analysis of the results of 161 persons receiving two doses 4 weeks apart and 61 persons receiving two doses 1 week apart. These groups were then sufficiently large for more detailed analysis.

The main question under consideration was the comparative degree of antigenic stimulation provided by initial immunization with two doses separated by different numbers of weeks. It was considered that the most meaningful comparison would be the circulating antibody levels at equivalent times after the second dose, when both groups had completed their so-called initial immunization and had received the same total amount of antigen. With the bleeding schedule that was employed, it was possible to compare these groups at the time of receipt of their second dose and 1, 2, and 6 weeks thereafter. Table 2 gives the Ogawa and Inaba whole-group geometric mean titers at the indicated times after the second injection, and the differences between the means. Very significantly higher vibriocidal antibody levels against both Ogawa and Inaba cells were found for the group receiving their two doses 1 week apart.

Work done under the previous contract had demonstrated that approximately 47% of the volunteers in our program had circulating vibriocidal antibody to one or both cholera serotypes prior to entering our study, and that individuals with preimmunization vibriocidal titers responded to immunization significantly better. The combined groups compared above contained sufficient numbers of individuals with antibodies detectable for neither serotype, or antibodies detectable for both serotypes, to permit statistically meaningful analyses of the influence of preimmunization titers upon serological response to the two different dosage schedules under consideration. Table 3A shows the geometric mean Ogawa and Inaba responses of individuals having undetectable preimmunization titers. The following table (Table 3B) shows similar information for persons having preimmunization titers against both Ogawa and Inaba serotypes. From these data it is apparent that the superiority of immunization using a 1-week interval is produced primarily by the favorable effect of this regimen upon individuals without detectable preimmunization antibody. Among individuals with preimmunization antibody there was no significant difference between the two immunization regimens in respect to Ogawa response, and the advantage of two doses a week apart in producing an anti-Inaba response was considerably less than with individuals without preimmunization titers.

In previous investigations there was clear evidence that although the administration of a second dose of vaccine given 4 weeks after the first did not produce a booster response, it did decrease the frequency of very low titers when compared with one-dose immunization. There were too few persons in the group receiving two doses a week apart to permit a comparison of the 1 week and 4 week interval between doses upon the frequency of poor responders. There are now a sufficient number of persons who have received immunization by these two schedules that this can be done. Figure 2 shows the cumulative percentage distributions of maximum anti-Ogawa and anti-Inaba vibriocidal titers (regardless of time of occurrence) among 71 persons receiving two doses 4 weeks apart and 28 persons injected at the 1-week interval. All of these persons had no detectable preimmunization vibriocidal antibody and therefore would be expected to be the group most refractory to immunization. This figure demonstrates the relatively smaller proportions of lower titers against both serotypes among the persons receiving two doses 1 week apart.

The results given here concerning the greater antibody response following two doses of cholera vaccine given a week apart, compared to that from equivalent administration separated by 4 weeks, appear to confirm and extend the tentative conclusions derived from our previous experiments. With the larger number of persons now available, it is now clear that two doses at a 1-week interval induce antibody formation over the initial 10 weeks of observation that equals or exceeds that produced by the 4-week regimen. If these vaccination procedures are compared in terms of their responses over the 6 weeks following the second dose, the differences are quite considerable on the whole-group basis. However, further analysis now indicates that individuals without pre-existing vibriocidal antibody are the ones who benefit most from the regimen in which initial immunization is given by two doses 1 week apart. The results of the continuing study of these groups through the full initial immunization cycle will become available this winter, and it is believed that there will be data from a sufficiently large number of volunteers to permit firm recommendations concerning the dosage interval to be used in initial immunization.

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2. Mosley, W. H. (1969) Texas Reports on Biol. and Med. 27:Suppl. 1, 227-241.

TABLE 1

Flow Chart of the Distribution of Volunteers into Groups and the Injection and Bleeding Schedule for the Groups

Group Designation			1	2	3	4	Bleeding Designation
Number of Volunteers			41	41	41	41	
Date	Project Time						
	Year	Week					
9/28/68	--	-2	B + E	B + E	B + E	B + E	B0
10/12/68	1	0	I	I	I	I	
10/19/68	1	1	B	B	B	B + I	B1
10/26/68	1	2	B	B	B	B	B2
11/2/68	1	3	B	B	B	B	B3
11/9/68	1	4	B + I	B + I	B + I	B	B4
11/16/68	1	5	B	B	B	B	B5
11/23/68	1	6	B	B	B	B	B6
11/30/68	1	7				B	B7
12/21/68	1	10	B	B	B	B	B8
4/12/69	1	26	B + I	B	B	B	B9
4/19/69	1	27	B				B10
4/26/69	1	28	B				B11
5/3/69	1	29	B				B12
5/24/69	1	32	B				B13
10/11/69	1	52	B + I	B + I	B	B	B14
10/18/69	2	1	B	B			B15
10/25/69	2	2	B	B			B16
11/1/69	2	3	B	B			B17
11/22/69	2	6	B	B			B18
4/11/70	2	26	B + I	B	B	B	B19
4/18/70	2	27	B				B20
4/25/70	2	28	B				B21
5/2/70	2	29	B				B22
5/23/70	2	32	B				B23
10/10/70	2	52	B + I	B + I	B + I	B + I	B24
10/17/70	3	1	B	B	B	B	B25
10/24/70	3	2	B	B	B	B	B26
10/31/70	3	3	B	B	B	B	B27
11/21/70	3	6	B	B	B	B	B28

B = Bleeding

E = Examination

I = Vaccine Injection
(All 0.5 ml except at
0:1 week and 0:4 week)

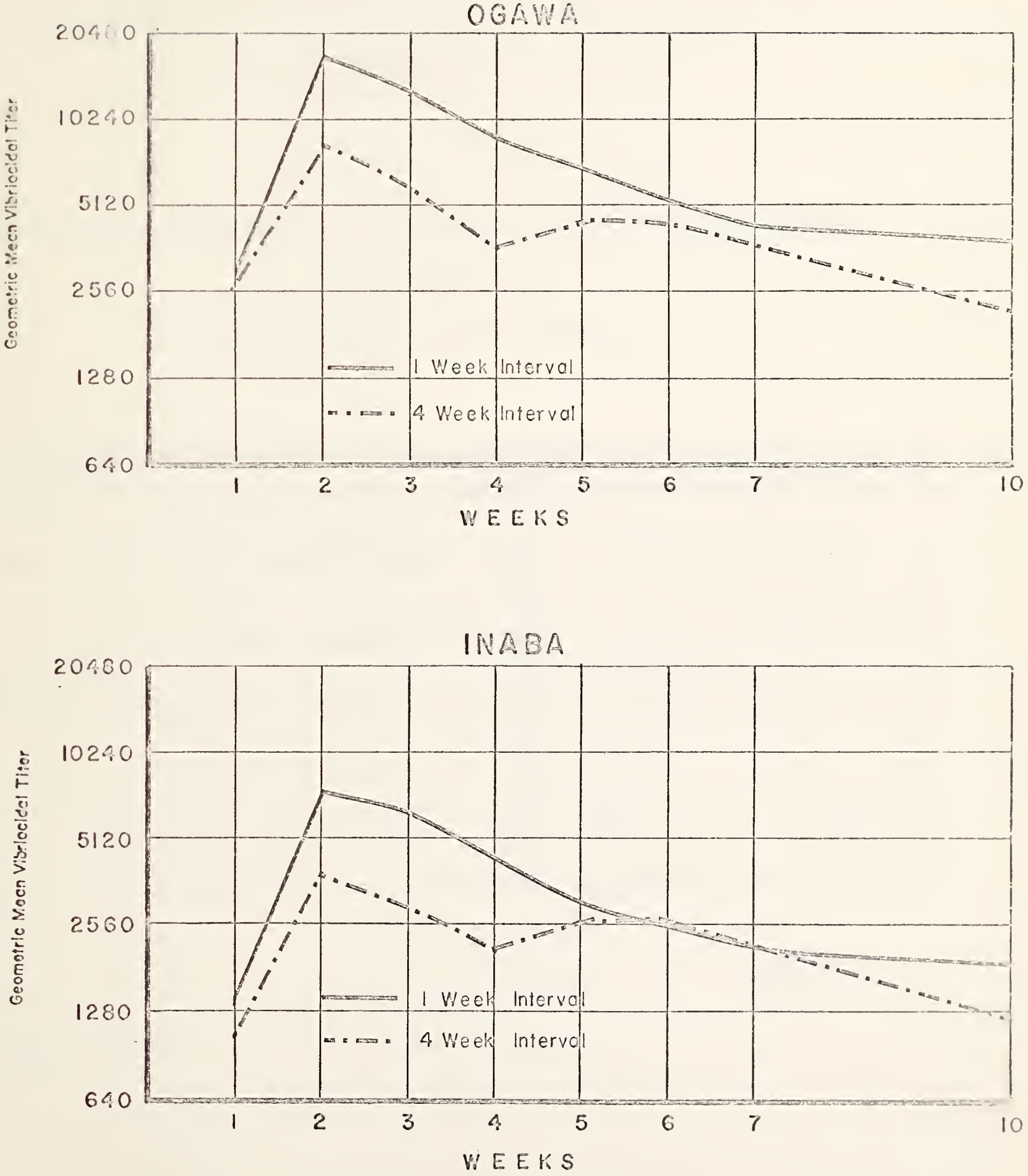

FIGURE 1. THE VIBRIOCIDAL RESPONSES OF VOLUNTEERS RECEIVING INITIAL IMMUNIZATION WITH TWO DOSES OF CHOLERA VACCINE AT INTERVALS OF 1 WEEK AND 4 WEEKS

TABLE 2

Whole-Group Geometric Mean Vibriocidal Titers after the Second Initial Immunization Dose

(Means Expressed as Tube Numbers)

Interval Between Doses	Anti-Ogawa Geometric Means				Anti-Inaba Geometric Means			
	Time after 2nd Dose				Time after 2nd Dose			
	0	1 week	2 weeks	6 weeks	0	1 week	2 weeks	6 weeks
1 week	11.05	13.43	13.07	11.53	9.95	12.45	12.24	10.63
4 weeks	11.46	11.69	11.60	10.63	10.65	11.14	10.98	9.96
Difference	-0.39	1.74*	1.47*	0.90*	-0.70*	1.31*	1.26*	0.67*

Tube 9 = 1:640

Tube 10 = 1:1280

Tube 11 = 1:2560

Tube 12 = 1:5120

Tube 13 = 1:10240

Tube 14 = 1:20480

*Significant Difference

TABLE 3A

Geometric Mean Vibriocidal Titers after the Second Initial Immunization Dose of Persons Having Negative Preimmunization Titers

(Means Expressed as Tube Numbers)

Interval Between Doses	Anti-Ogawa Geometric Means				Anti-Inaba Geometric Means			
	Time after 2nd Dose				Time after 2nd Dose			
	0	1 week	2 weeks	6 weeks	0	1 week	2 weeks	6 weeks
1 week	10.66	13.09	12.79	11.21	9.11	12.16	12.07	10.36
4 weeks	11.04	11.29	11.31	10.24	9.39	10.36	10.41	9.39
Difference	-0.38	1.80*	1.48*	0.97*	-0.28	1.80*	1.66*	0.97*

TABLE 3B

Geometric Mean Vibriocidal Titers After the Second Initial Immunization Dose of Persons Having Preimmunization Vibriocidal Antibody Against both Inaba and Ogawa Serotypes

(Means Expressed as Tube Numbers)

Interval Between Doses	Anti-Ogawa Geometric Means				Anti-Inaba Geometric Means			
	Time after 2nd Dose				Time after 2nd Dose			
	0	1 week	2 weeks	6 weeks	0	1 week	2 weeks	6 weeks
1 week	10.69	13.17	12.83	11.54	11.27	13.13	12.71	11.29
4 weeks	12.20	12.53	12.38	11.62	11.28	11.67	11.59	10.60
Difference	-1.51*	0.64	0.45	-0.08	-0.01	1.46*	1.12*	0.69

Tube 9 = 1:640
Tube 10 = 1:1280
Tube 11 - 1:2560

Tube 12 = 1:5120
Tube 13 = 1:10240
Tube 14 = 1:20480

*Significant Difference

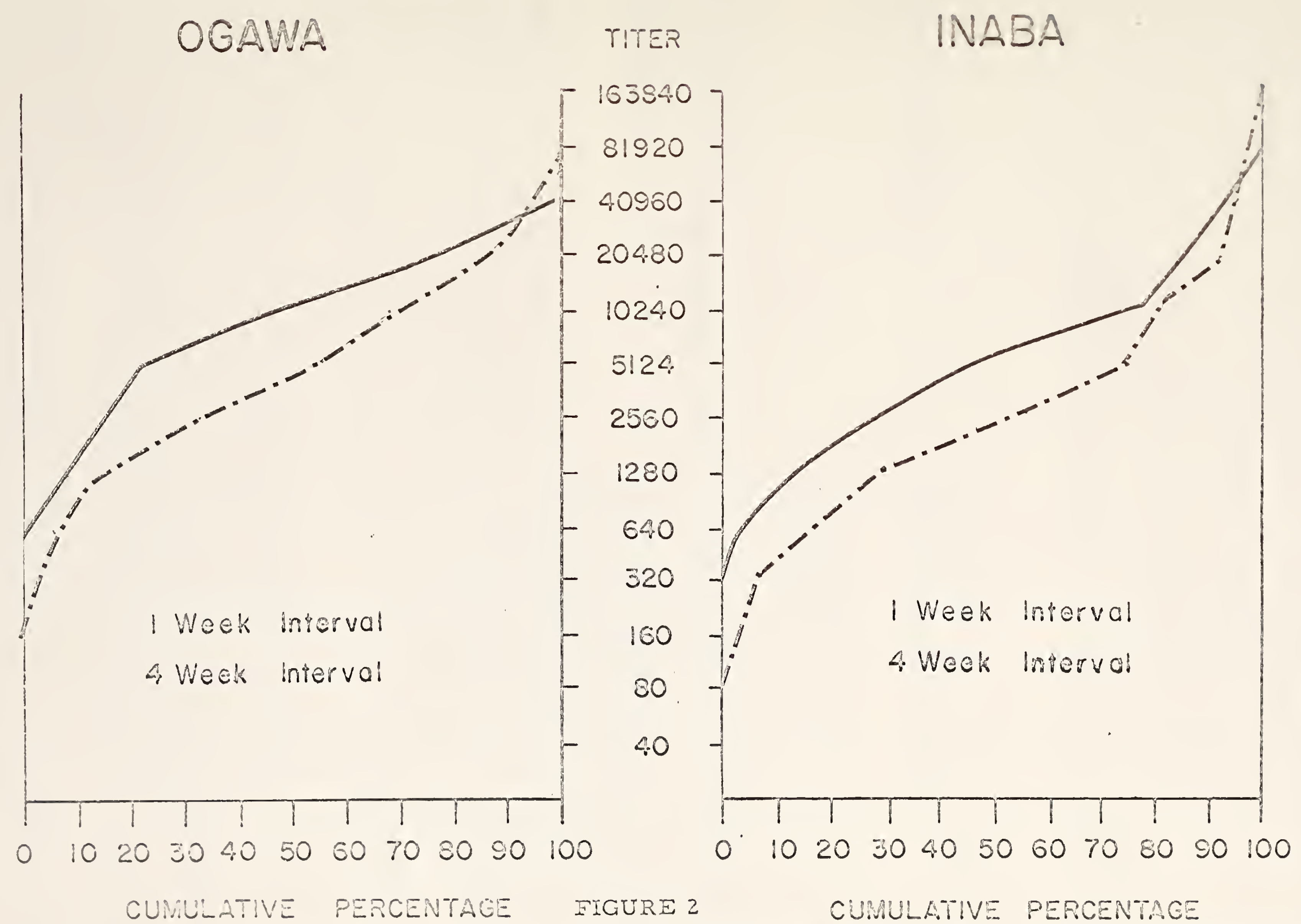

FIGURE 2

CUMULATIVE PERCENTAGE DISTRIBUTION OF MAXIMUM VIBRIOCIDAL TITERS (REGARDLESS OF TIME OF OCCURRENCE) IN PERSONS WITHOUT DETECTABLE PREIMMUNIZATION ANTIBODY WHO HAVE RECEIVED TWO DOSES OF CHOLERA VACCINE AT 1 WEEK OR 4 WEEK INTERVAL

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13. ABSTRACT

This study of the vibriocidal response to initial immunization and reimmunization with cholera vaccine employs four groups of volunteers, three of which have been immunized with two doses 4 weeks apart, and one which received two weekly doses. The three groups will be reimmunized at intervals of 6 months, 1 and 2 years; and the group immunized at the 1-week interval will be reimmunized at 2 years.

No data are yet available concerning reimmunization. However, comparisons of response to initial immunization with two doses at 1-week or 4-week intervals have resulted in the following findings:

1. In confirmation of results obtained under a previous contract, the response to initial immunization following the second dose at 4 weeks is generally lower than the response to the first dose and no booster effect is seen.
2. Initial immunization consisting of two doses given at a 1-week interval, produces group mean titer responses that are at least equivalent to or (at most points) higher than the responses obtained with two doses given 4 weeks apart.
3. The comparison of group geometric mean titers 1, 2, and 6 weeks after the second dose demonstrates a very significant difference in favor of two doses given at weekly intervals.
4. The use of two doses 1 week apart is of greatest advantage in the immunization of persons who do not have pre-existing vibriocidal antibody. These persons show higher group mean titers and a decreased frequency of poor responses compared to those immunized with two doses 4 weeks apart.

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	ROLE	WT	ROLE	WT	ROLE	WT
Cholera Vaccine						
Immunity						
Serological response						
Human						
Initial immunization						
Reimmunization						
Interval						
Natural immunity						

AD________________

Normal Human Serum (U)

FINAL REPORT

by

Ralph J. Wedgwood, M.D.

July 1969
(For the period 1 August 1962 to 31 July 1967

U.S. Army Medical Research and Development Command

Washington, D.C. 20315

Final Report to the Commission on Immunization
of the Armed Forces Epidemiological Board

Contract No. DA-49-193-MD-2308

University of Washington

Seattle, Washington 98105

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SUMMARY

Three distinct somatic antigens of *Vibrio cholera* were identified: one specific for Ogawa, one for Inaba, one common to both. Hikojima strains contained all 3 antigens. Selection of an appropriate Hikojima strain might allow the development of a single immunizing agent, effective against all strains.

Spheroplasts of *Vibrio cholera* were produced in serum, and L-form type colonies were grown on special media. The replicative particle passed through 0.45μ filters.

A sequence for the bactericidal action of serum is proposed: Using R for bacterial rod, R^L for the lysozyme-sensitive rod, R^* for nonreplicative or killed rod, S for spheroplast and G for ghost:

$$\begin{tikzcd} R \arrow[r, shift right] & R^L \arrow[l, shift right, "1"'] \arrow[r, "2"] \arrow[rr, bend left, "5"] & R^* \arrow[r, "3"] & S \arrow[r, "4"] & G \end{tikzcd}$$

Studies in an experimental animal, *Siredon mexicanum*, and in patients with immunologic defects, suggest independence of IgG and IgM antibody responses, and independence of antibody response and immunoglobulin synthesis from morphologic organization of lymph nodes. The primacy of the GI tract in IgA synthesis is suggested.

Appended is a list of 17 published articles and 7 published abstracts derived from work conducted in whole or in part under this contract.

Foreword

Submitted herewith is the final report for Research Contract No. DA-49-193-MD-2308 covering the period, 1 August, 1962 through 31 July, 1967, the termination date of the contract.

The majority of the work conducted through this contract has now been published. The information contained in these publications, which have been previously submitted to the A.F.E.B., because it is available in printed form, is not included in this report. A complete bibliographic listing of 17 articles and 7 abstracts is provided. Information not yet published is summarized and presented.

Those findings with potential major significance to the Armed Forces are identified in the summary. Suggestions for future approaches to problems of both civilian and military importance are included.

The support of the A.F.E.B. over many years of continuous association has been appreciated, as has the stimulus and interest of members of the Commission on Immunization.

The work reported includes research performed by the following:

Edwin S. Boatman
John T. Boyer
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Starkey D. Davis
H. Diethard Gemsa
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David B. Shurtleff
Earl W. Todd
Marvin Turck
Christopher P.S. Williams
Richard H. Winterbauer

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council.

REPORT

Cholera remains an important problem of world health and a significant problem for the Armed Forces. The work reported here was initiated in 1960, with the intent of providing information on the somatic antigens of *Vibrio cholera* for improved vaccine production. The studies were carried out at the Microbiological Research Laboratory, Porton, England (1960-61); at Western Reserve University, Cleveland, Ohio (1961-62); and thereafter at the University of Washington, Seattle. While this report officially covers only the period 1963 to 1968, the prior work is reviewed to provide necessary background.

Preparation of Materials

Five standard strains of *Vibrio cholera*, Ogawa NCTC 5596, Inaba NCTC 4693, El Tor NCTC 8457, RDE NCTC 8367, and Hikojima NCTC 7270 were obtained and used for all studies. Large batch cultures were made in minimal liquid media. The organisms were acetone killed, washed, and freeze-dried. Lipopolysaccharide was prepared by phenol-water extraction (Westphal technique). The lipopolysaccharide was repeatedly washed and extracted to remove nucleic acid and other contaminants, then freeze-dried.

Chemical Characterization of Lipopolysaccharides

The composition of the lipopolysaccharides is tabulated below. There was remarkable uniformity between strains.

TABLE I

	RDE	Ogawa	Inaba	Hikojima	El Tor
Nitrogen	3	3	3	4	4
Amino acids less hexosamic	12	9	12	13	16
Hexosamic	9	6	9	9	9
Nucleic acid	2	2	2	6	2
Total sugar	31	22	32	33	36
?Heptose	14	10	18	15	14
Lipid (approximate)	50-55	50-55	50-55	50-55	50-55

Estimates of the composition of lipopolysaccharide from various strains of *Vibrio cholera*, expressed as percent dry weight.

Analysis for unusual sugars was attempted. Using the Dische cysteine sulfuric acid technique, an unusual peak, at 510 mμ was found constant with the presence of a heptose. However, on repeated attempts at separation on chromatography, this type of sugar could not be identified or isolated. It is possible that acid-labile sugars were destroyed. The lipopolysaccharides were all highly resistant to mild acid hydrolysis. Standard techniques with hydrochloric acid could have denatured labile sugars. Mild techniques, with N/10 Acetic acid required up to 24 hours for splitting of lipid from polysaccharide. Even in a nitrogen atmosphere such periods might well destroy some unusual sugar components. The most probable composition with these limitations is shown in Table II.

TABLE II

Probable Sugars Present

Hexosamine--probably glucosamine
Glucose
Glucuronic acid
Galactose

Preparations of lipid produced by mild hydrolysis were also studied. These lipids contained less than 5% residual sugar. The lipid was quite soluble in ether and chloroform, and even more soluble in ether-alcohol or chloroform-alcohol mixtures. These characteristics are most compatible with a highly polar lipid compound. Further fractionation of the lipids was possible by differential solubility in petroleum ether, benzene and chloroform. However, reliable quantitative data could not be obtained because of the limited amounts of material available. Silicic acid chromatography could not be accomplished because of inadequate solubility.

Antigenic Analysis by Double Diffusion

Antisera were produced in rabbits by the injection of whole bacteria, and on occasion, stimulation with lipopolysaccharide. Reference antisera were also obtained from Kaufmann. Using whole bacteria, lipopolysaccharide, native antisera, adsorbed antisera and antigen inhibition, the following antigenic analysis for the somatic antigens was identified.

TABLE III

Strains	Antigens A	B	C
Ogawa	+	+	
Inaba	+		+
Hikojima	(+)	+	+

This antigenic analysis is in agreement with that of Kaufmann. There appear to be two distinct strain specific antigens, one identified with Ogawa strain, the other with Inaba strains. There was also a common antigen, found in all strains. Hikojima strains apparently contained all three antigens, although the presence of the common antigen by the nature of the test system could not be absolutely confirmed. El Tor strains of both Inaba and Ogawa types were identified.

Development of a Bactericidal Assay

The most sensitive immunologic technique available is the killing of bacteria by specific antibody and complement. This technique provides an excellent assay for antigens by use of antigen inhibition.

To develop this test, consistent plating techniques for colony counting were necessary. Optional bacterial growth and colony counts were obtained by using Brain Heart Infusion and Blood Agar Base, to which approximately 2 gm/liter salt and glucose were added. The pH was adjusted to 7.6-7.8 with 1 M. Tris. It is of interest that in this simple liquid media bacterial concentrations as high as 10^{11}/ml. could be obtained, and on the solid media discretely and well.

A two-phase bactericidal assay was developed. A log-phase bacterial suspension (washed, suspended in saline) was added in the cold to a dilution of antiserum. The bacteria were then either washed, or the excess antiserum eliminated by dilution, and an aliquot was plated out on solid media. After overnight incubation, colonies were counted. Control counts included zero time samples, and suspensions incubated without antibody, and without complement. The percent killing was plotted and a 50% end point determined. The technique was highly reproducible, extremely sensitive, and apparently accurate.

Antigenic Analysis by Bactericidal Assay

By cross-adsorption with whole bacteria, monospecific antisera were developed. Using cross-adsorption and testing with both homologous and heterologous strains, the antigenic determinants by this assay appeared identical to those demonstrated by the Ouchterlony technique. Using carefully selected antisera, with the appropriate bacterial strain and a complement source adsorbed to remove all demonstrable antibody activity, a separate assay system could be set up for each of the three identified antigens. The common antigen can be determined by using mismatched antiserum and test organisms; for example, Ogawa organisms in Inaba antisera.

The test system proved to be specific, reliable, and highly sensitive. The data obtained confirmed entirely the postulated antigenic composition shown in Table III. El Tor strains antigenically resembling both Inaba and Ogawa strains were found.

Antigenic Analysis by Hapten Inhibition

The two-stage bactericidal assay lends itself well to analysis of antigenic components by hapten inhibition. For this purpose, varying amounts of the material to be tested were added to the antiserum dilution; the mixture was allowed to stand in the cold, precipitated removal by centrifugation, and then the residual antibody was tested for activity.

The antigenic composition of the five lipopolysaccharides was determined quantitatively by this technique. The results from a large number of comparable experiments are summarized in Table IV.

TABLE IV

Antigenic Components of Lipopolysaccharide from Five Strains of _Vibrio_ _Cholera_

Source of lipopolysaccharide	Antigenic Composition* (Half log approximation) A	B	C
RDE strain (NCTC 8367)	33	100	0**
Ogawa strain (NCTC 5596)	33	100	0**
Hikojima strain (NCTC 7270)	100	1	100
Inaba strain (NCTC 4693)	33	0**	33
El Tor strain (NCTC 8457)	33	0**	33

*Reciprocal of amount of lipopolysaccharide in mcg giving 50% inhibition in specific bactericidal assay system.

**Less than 0.1

In every experiment the assay system reacted as if these three antigens were the only significant determinants. While additional antigens may be present, using this highly sensitive methodology they could not be demonstrated.

The data have more than merely theoretical interest. Previously, we and others have questioned the existence of both Inaba and Ogawa specific antigens in Hikojima strains. The data, using this highly sensitive test system, consistently demonstrate that this is the case, and previous variance in reports may well relate to quantitative rather than qualitative differences. The Hikojima strain studied here, while more "like" the Inaba strain, still definitely also contains Ogawa specific antigen. Secondly, in our laboratory, the clear demonstration of a common somatic antigenic determinant in all strains tested suggests the possibility that a considerable degree of common immunity to all strains of _Vibrio_ _cholera_ could exist as a resistance mechanism.

While haptenic activity cannot be directly translated into terms of antigenic activity, if antibodies to the somatic (lipopolysaccharide) antigens of *Vibrio cholera* play any role in natural or induced resistance to disease in man, these data suggest that both through immunizing for a common antigen and for immunizing for Inaba or Ogawa specific antigens, a single carefully selected Hikojima strain could theoretically be used to induce simultaneous immunity to all three antigens: the common antigen (A), and the two specific antigens Ogawa (B) and Inaba (C). Thus, theoretically, it can be suggested that a Hikojima strain selected on this basis might provide a single, effective polyvalent immunizing agent. The practical implications of this suggestion need no elaboration.

Haptenic Activity of the Lipid Moiety

Studies were carried out on the fractionation of lipopolysaccharide to determine where the haptenic determinant resided. This work was made difficult and tedious by three problems. First, the lipopolysaccharides, particularly of the Ogawa strains, were extremely resistant to hydrolytic splitting of the lipid and polysaccharide moieties. Second, the lipid so derived was difficult to handle and all dispersing agents tested interfered with the assay technique. Finally, the recoverable yield of the lipid was extremely small.

Nevertheless, using prolonged (24 hours or more) mild hydrolysis in 0.1 N Acetic acid we were able to split the lipopolysaccharide into two moieties. One was a classic polysaccharide, easily soluble in water, making a clear, syrupy solution. This fraction was uniformly completely devoid of *any* haptenic activity in the bactericidal system.

The lipid moiety was a typical "fatty" substance, which was soluble in chloroform. The best preparations contained no more than 5-10% polysaccharide (Orcinol reactive material). A suspension could be made in aqueous solutions from the chloroform dissolved material, and the chloroform could be removed sufficiently for testing. However, the quantitation of hapten inhibition in the bactericidal assay was possibly inaccurate since the lipid aggregated rapidly, in spite of emulsification by ultrasonic agitation. Thus, the actual activity may be far greater than that measured.

In spite of these problems, the data appear to be sufficient for some preliminary conclusions. These data are summarized in Table V.

TABLE V

	Antigenic Composition (mcg for 50% inhibition)		
Source of Lipid	A	B	C
RDE (NCTC 8367)	No activity ⟶		
Ogawa (NCTC 5596)	No activity ⟶		
Hikojima (NCTC 7270)	250	No activity	250
Inaba (NCTC 4693)	180	No activity	180
El Tor (NCTC 8457)	500	No activity	250

No activity equals no inhibition at concentrations of >2000 mcg/ml

It is quite apparent that there are fundamental differences between the Ogawa-type and the Inaba-type lipids. It would appear that haptenic activity of the lipopolysaccharide of the RDE and Ogawa strains is in large part destroyed by hydrolysis. The Inaba-type lipid retains significant activity. These suggested chemical differences would be consistent with the differences reported elsewhere by Verwey, using a different test system. In both laboratories the data are consistent with the hypothesis that the lipid moiety is an important factor in the antigenic (haptenic) determinant. Likewise, the findings in both laboratories suggest that there are distinctive differences, possibly related to macromolecular configuration, between these two prototype antigens.

The data do not allow a definite conclusion as to the nature of the antigenic determinant as a lipid. The residual polysaccharide in the lipids produced would be sufficient to constitute the determinant grouping--carried on a lipid molecule. However, the complete absence of activity in the polysaccharide moiety does make the suggested importance of the lipid in this regard the most attractive hypothesis.

Attempts at further fractionation of the lipid were limited by the minimal amounts of material available. Column fractionation on Silicic Acid could not be performed because of aggregation of the lipid. We could, however, further isolate the activity through differential solubility. The activity of the Inaba and El Tor lipid resided primarily in a fraction that was insoluble in Petroleum Ether or Benzene. The Petroleum Ether soluble fraction had virtually no activity. The Benzene soluble fraction retained some, but limited, activity. These data suggest that the activity resides primarily in the polar lipid fraction.

Other Work

Work on spheroplasts of *Vibrio cholera*, or the bactericidal action of serum and on the ontogeny of immunity carried out under the auspices of this contract have been published. Copies of reprints have been previously submitted and the articles are listed in the appended bibliography. It would be redundant to describe the work further in this final report, and readers are referred to the articles for the needed details.

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*Although the work reported in this publication was completed subsequent to termination of the grant, we have included it in the bibliography because it derived from work carried out under the auspices of the grant.

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13. ABSTRACT

Three distinct somatic antigens of Vibrio cholera were identified: one specific for Ogawa, one for Inaba, one common to both. Hikojima strains contained all 3 antigens. Selection of an appropriate Hikojima strain might allow the development of a single immunizing agent, effective against all strains.

Spheroplasts of Vibrio cholera were produced in serum, and L-form type colonies were grown on special media. The replicative particle passed through 0.45 μ filters.

A sequence for the bactericidal action of serum is proposed: Using R for bacterial rod, R^L for the lysozyme-sensitive rod, R* for nonreplicative or killed rod, S for spheroplast and G for ghost:

$$\begin{tikzcd} R \arrow[r, shift left, "1"] & R^L \arrow[l, shift left] \arrow[r, "2"] \arrow[rr, bend left, "5"] & R^* \arrow[r, "3"] & S \arrow[r, "4"] & G \end{tikzcd}$$

Studies in an experimental animal, Siredon mexicanum, and in patients with immunologic defects, suggest independence of IgG and IgM antibody responses, and independence of antibody response and immunoglobulin synthesis from morphologic organization of lymph nodes. The primacy of the GI tract in IgA synthesis is suggested.

Appended is a list of 17 published articles and 7 published abstracts derived from work conducted in whole or in part under this contract.

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